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(54) Title: METHODS AND MATERIALS FOR TREATING HUMAN PAPILLOMAVIRUS INFECTIONS

(57) Abstract: The invention provides methods and materials related to treating HPV infections (e.g., HPV infections of cutaneous and mucosal epithelial cells) and HPV-associated conditions (e.g., cervical dysplasia, HPV-associated cervical carcinomas, oral mucosal papilloma cancers, laryngeal papilloma cancers).

## METHODS AND MATERIALS FOR TREATING HUMAN PAPILLOMAVIRUS INFECTIONS

### Related Applications

5           This application claims the benefit of U.S. Provisional Patent Application Serial No. 60/391,795, filed June 26, 2002; U.S. Provisional Patent Application Serial No. 60/417,997, filed October 14, 2002; and U.S. Provisional Patent Application Serial No. 60/449,066, filed February 21, 2003.

### Statement as to Federally Sponsored Research

10           Funding for the work described herein was provided by the federal government, which may have certain rights in the invention.

## BACKGROUND

### 15    1. *Technical Field*

          The invention relates to methods and materials involved in treating human papillomavirus infections and human papillomavirus-associated conditions.

### 2. *Background Information*

20           Human Papillomaviruses (HPV), members of the papillomavirinea family of viruses, are recognized as a human pathogen. Various types of HPV are tropic for cutaneous and mucosal epithelial cells, and consequently they are involved in development of papillomas (including common, genital, and planter warts), and cervical carcinomas (zur Hausen, *Curr. Top. Microbiol. Immunol.*, 186:131-56 (1994)). Types 16,  
25   18, 31, 33, 35, 39, 45, 52, and 58 are involved in nearly 100 percent of all carcinomas of the cervix (Walboomers *et al.*, *J. Pathol.*, 189:12-9 (1999)), with 16 and 18 constituting "high risk" types.

## SUMMARY

30           The invention provides methods and materials related to treating HPV infections (e.g., HPV infections of cutaneous and mucosal epithelial cells) and HPV-associated

conditions (e.g., cervical dysplasias (also referred to as cervical intraepithelial neoplasias), HPV-associated cervical carcinomas, oral mucosal papilloma cancers, and laryngeal papilloma cancers). For example, the invention provides (1) methods for treating a mammal having cells infected with HPV, (2) antisense molecules, small  
5 interfering RNA (siRNA) molecules, DNazymes (Dz), and ribozymes (Rz) having a sequence complementary to HPV sequences (e.g., RNA), (3) methods for making antisense molecules, siRNA molecules, Dz, and Rz having a sequence complementary to HPV sequences, (4) methods for obtaining a library of single-stranded Dz, (5) methods for identifying HPV target sequences, (6) methods for treating HPV infections and HPV-  
10 associated conditions, and (7) methods and materials for delivering antisense molecules, siRNA molecules, Dz, and Rz to cells infected with HPV.

The invention is based, in part, on the discovery that nucleic acid molecules such as antisense molecules, siRNA molecules, Dz, and Rz can be used successfully to reduce the number of HPV infected cells in a mammal. For example, antisense oligonucleotides  
15 (ASO) and Dz designed to interact with HPV E6/E7 RNA can be used to reduce the number of HPV infected cells within a mammal. The HPV infected cells can be cells containing non-integrated HPV nucleic acid and can be exhibiting HPV replication. As described herein, nucleic acid molecules were successfully used to reduce the number of HPV infected cells in a mammal. For example, library selection protocols were  
20 developed and used to identify accessible sites in E6/E7 target RNA from HPV11, an HPV type implicated in causation of genital warts. ASO, Rz, and Dz were designed to contain sequences complementary to the identified sites. In addition, the ASO, Rz, and Dz were tested in cell culture. Further, ASO and Dz were used topically to treat HPV11-infected human foreskin grafted onto immunodeficient mice. Topical administration of  
25 ASO and Dz resulted in a reduction in the development of HPV infections. In addition, an ASO targeting a site, which had been identified in two different library selection protocols, exhibited the greatest efficacy. For example, antisense molecules targeted to the region surrounding one of the library-selected sites (e.g., nucleotide 407) proved extremely effective in blocking development of HPV11-induced papillomas in human  
30 foreskin grafts on immunodeficient mice. The results provided herein demonstrate that

molecular therapeutics (e.g., antisense molecules, siRNA molecules, Rz, and Dz) can be used to treat bona fide HPV infections *in vivo*.

In general, the invention features a method of treating HPV infection comprising administering an effective amount of a nucleic acid molecule to a patient in need thereof  
5 wherein the nucleic acid molecule inhibits expression associated with HPV replication. The inhibition can be at any level (e.g., 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or more percent inhibition). The HPV can be HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV52, or HPV58. The nucleic acid molecule can be complementary to a nucleic acid sequence of the HPV. The nucleic acid  
10 molecule can be administered topically. The nucleic acid molecule can be administered topically to a portion of the genital organ of the patient. The nucleic acid molecule can be administered topically to cervical tissue of the patient. The nucleic acid molecule can be an antisense oligonucleotide or an siRNA molecule. The nucleic acid molecule can be a ribozyme or DNAzyme. The nucleic acid molecule can be administered together with a  
15 keratolytic agent, wherein the keratolytic agent can be present in an amount effective to enhance the penetration of the nucleic acid molecule. The keratolytic agent can be salicylic acid or one or more alpha hydroxy acids. The patient can suffer from cervical intraepithelial dysplasia (CIN). The CIN can be CIN I or mild dysplasia. The CIN can be CIN II or moderate to marked dysplasia. The CIN can be CIN III or severe dysplasia to  
20 carcinoma-in-situ. The carcinoma-in-situ can be localized to the intraepithelial tissue or the superficial layer of the cervix. The nucleic acid molecule can be identified using a library selection technique. The library selection technique can be an antisense oligonucleotide library selection technique, a ribozyme library selection technique, or a DNAzyme library selection technique.

25 In another embodiment, the invention features a method for treating a mammal having cells infected with HPV. The method includes administering a nucleic acid molecule to the mammal under conditions wherein the number of the cells infected with the HPV is reduced, wherein the nucleic acid molecule contains a sequence complementary to a nucleotide sequence of the HPV. The mammal can be a non-human,  
30 immunodeficient mammal, and the cells can be human cells. The mammal can be a nude or SCID mouse. The mammal can be a human. The cell can be a skin cell or epithelial

cell. The HPV can be HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV52, or HPV58. The nucleic acid molecule can be administered topically to the mammal. The nucleic acid molecule can be administered topically to a portion of the genital organ of the mammal. The nucleic acid molecule can be administered topically to cervical tissue of the mammal. The nucleic acid molecule can be an antisense oligonucleotide or an siRNA molecule. The nucleic acid molecule can be a ribozyme or DNAzyme. The number of the cells infected with the HPV can be reduced by at least 25 percent, at least 50 percent, or at least 75 percent. The nucleotide sequence can contain a target site identified using a library selection technique. The library selection technique can be an antisense oligonucleotide library selection technique, a ribozyme library selection technique, or a DNAzyme library selection technique. The cells infected with the HPV can contain non-integrated HPV nucleic acid. The cells infected with the HPV can contain replicating HPV.

In another aspect, the invention features an isolated catalytic nucleic acid containing a catalytic core sequence, a 5' recognition sequence, and a 3' recognition sequence, wherein the isolated catalytic nucleic acid cleaves a target mRNA sequence selected from the group consisting of the sequences set forth in SEQ ID NOs:4-48. The isolated catalytic nucleic acid can be a ribozyme. The catalytic core sequence can contain the sequence set forth in SEQ ID NO:51. The isolated catalytic nucleic acid can be a DNAzyme. The catalytic core sequence can contain the sequence set forth in SEQ ID NO:68.

In another embodiment, the invention features an isolated, single-stranded nucleic acid molecule consisting of between five and 40 nucleotides, wherein the nucleic acid molecule contains a sequence complementary to at least five consecutive nucleotides of a sequence selected from the group consisting of SEQ ID NOs:4-48. The nucleic acid molecule can be DNA.

Another embodiment of the invention features an isolated, double-stranded RNA molecule, wherein one strand of the RNA molecule contains a nucleic acid sequence complementary to at least five consecutive nucleotides of a sequence selected from the group consisting of SEQ ID NOs:4-48. The RNA molecule can consist of between five and 40 nucleotides. The RNA molecule can consist of between 18 and 25 nucleotides.

Another aspect of the invention features a method for making a library of single-stranded DNAzymes. The method include (a) amplifying nucleic acid in an amplification reaction to form an amplification reaction product, wherein the amplification reaction contains template nucleic acid and a primer containing at least one ribose nucleotide, wherein one strand of the template nucleic acid contains the library of single-stranded DNAzymes, and wherein the amplification reaction product contains double-stranded nucleic acid with a strand containing the at least one ribose nucleotide, (b) contacting the amplification reaction product with a base hydrolysis agent under conditions wherein the strand containing the at least one ribose nucleotide becomes shorter than the other strand of the double-stranded nucleic acid, and (c) obtaining the strand from step (b) containing the library of single-stranded DNAzymes based on size. The amplification reaction can further contain a primer lacking ribose nucleotides. The amplification reaction can contain two primers containing at least one ribose nucleotide. The shorter strand of the double-stranded nucleic acid of step (b) can contain the library of single-stranded DNAzymes. The longer strand of the double-stranded nucleic acid of step (b) can contain the library of single-stranded DNAzymes. The base hydrolysis agent can be sodium hydroxide.

Another aspect of the invention features a composition containing a nucleic acid molecule and a keratolytic agent, wherein the nucleic acid molecule contains a sequence complementary to a nucleic acid sequence present in an HPV, and wherein the keratolytic agent is present in an amount effective to enhance penetration of the nucleic acid molecule. The nucleic acid molecule can be an antisense oligonucleotide or an siRNA molecule. The nucleic acid molecule can be a ribozyme or DNAzyme. The keratolytic agent can be salicylic acid.

Another aspect of the invention features a nucleic acid molecule containing  $(N)_{n1}$  followed by (oxygen-carbon-carbon) $_{n2}$  followed by  $(N)_{n3}$ , wherein N is a nucleotide, and wherein the  $n1$ ,  $n2$ , and  $n3$  are integers greater than 0. The  $n1$  can be between 10 and 20. The  $n2$  can be between 3 and 10. The  $n3$  can be between 10 and 20. The  $(N)_{n1}$  can be a DNA sequence of 17 consecutive adenosines. The  $(N)_{n3}$  can be a 5'-TGTAACGACGGCCAG-3' (SEQ ID NO:79) sequence. The nucleic acid molecule can contain a phosphate group between the (oxygen-carbon-carbon) $_{n2}$  and the  $(N)_{n3}$ .

Another aspect of the invention features a method for making a library of single-stranded DNAzymes. The method includes (a) amplifying nucleic acid in an amplification reaction to form an amplification reaction product, wherein the amplification reaction contains template nucleic acid and a primer containing an (oxygen-carbon-carbon)<sub>n</sub> backbone unit, wherein n is an integer greater than 1, wherein one strand of the template nucleic acid contains the library of single-stranded DNAzymes, and wherein the amplification reaction product contains double-stranded nucleic acid wherein the strand extending from the primer is shorter than the other strand of the double-stranded nucleic acid, and (b) obtaining the strand from step (a) containing the library of single-stranded DNAzymes based on size.

Another aspect of the invention features a method for reducing HPV replication in a cell, wherein the method includes contacting the cell with an antisense molecule, siRNA molecule, Dz, or Rz having sequence specificity for HPV RNA.

In another aspect, the invention features a method for inhibiting development of HPV in a mammal (e.g., human). The method includes (a) identifying via a library selection technique one or more target sites on HPV RNA that are accessible to antisense oligonucleotides, DNAzymes, or ribozymes; (b) designing one or more antisense oligonucleotides, siRNA molecules, DNAzymes, or ribozymes that are at least partially complementary to all or a portion of one or all of the target sites identified in step (a); and (c) contacting the mammal with one or more of the antisense oligonucleotides, siRNA molecules, DNAzymes, or ribozymes designed in step (b) such that development of HPV in the mammal is inhibited.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the

following detailed description, and from the claims.

### DESCRIPTION OF DRAWINGS

Figure 1 is a schematic overview of the LS3 (Dz selection library) method.

5 Figure 2 contains three panels related to the LS3 method. Panel A is a diagram of a PCR reaction used to generate double-stranded DNA, where one strand contains a library of Dz. The TS-17 (SEQ ID NO:1) and BS-17 (SEQ ID NO:2) primers are labeled. The amplified template (SEQ ID NO:3) contains the primer sequences and random nucleotide stretches flanking a catalytic core sequence (underlined). Panel B is a  
10 photograph of a sequencing gel of the PCR products of the Dz library. Panel C is a schematic of several amplification reactions using primers containing a ribose nucleotide (TS<sub>R</sub> and BS<sub>R</sub>) and 2-deoxyribose (TS<sub>D</sub> and BS<sub>D</sub>) primers. The thick lines pointing to the right represent strands containing active Dz sequences.

Figure 3 is a diagram of a nucleic acid molecule containing a carbon spacer.

15 Figure 4 contains photographs of gels separating cleavage products produced in an *in vitro* assay using the indicated Rz (A) and Dz (B).

Figure 5A is a diagrammatic representation of a SNIP<sub>AA</sub> expression cassette with the CLIP and CHOP portions indicated. S1 and S2 identify the autocatalytic cleavage sites for the cis-acting Rz in CLIP, while S3 and S4 identify the autocatalytic cleavage  
20 sites for the cis-acting Rz in CHOP. dITRz are depicted for the liberated trans-acting Rz, although single ITRz can be used instead of dITRz. A poly(A) tract is near the 3'-end of the liberated dITRz or ITRz in the SNIP<sub>AA</sub> cassette. The CHOP portion contains longer complementary flanking sequences for the cis-acting Rz, which enhances their autocatalytic activity. Figure 5B is a bar graph plotting the relative amount of HPV11  
25 E6/E7 mRNA in cells co-transfected with (1) a construct designed to express HPV11 E6/E7 target mRNA and (2) a construct designed to express an ITRz or dITRz targeting the indicated target site. Figure 5C is a bar graph plotting the relative amount of HPV11 E6/E7 mRNA in cells stably expressing HPV11 E6/E7 target mRNA and transfected with a Dz designed to target the indicate site.

30 Figure 6 contains two photographs of gels demonstrating *in vitro* cleavage of HPV16 E6/E7 RNA via Rz targeting library-selected sites. Panel A: 40 nM of the



individual Rz and 10 nM of 5'-end  $^{32}\text{P}$ -labeled HPV16 target RNA (782 nucleotides) were incubated for 30 minutes at 37°C in 20 mM Tris-HCl (pH 7.4) containing 25 mM  $\text{MgCl}_2$ . Panel B: 1, 2.6, 6.4, 16, 40, and 100 nM of Rz59 and 10 nM of 5'-end  $^{32}\text{P}$ -labeled HPV16 E6/E7 RNA (782 nucleotides) were incubated for 30 minutes at 37°C in 20 mM Tris-HCl (pH 7.4) containing 25 mM  $\text{MgCl}_2$ .

Figure 7 is a schematic diagram of the predicted structure of HPV16 E6/E7 mRNA as determined using mFold plot. The locations of the Rz sites identified by library selection are labeled as sRz sites, and those chosen on the basis of the mFold modeling are labeled as mRz.

Figure 8A is a photograph of a gel used to quantify the levels of HPV16 E6/E7 RNA in cells transfected with SNIP<sub>AA</sub>Rz constructs expressing the indicated Rz. The "AA" denotes an empty SNIP<sub>AA</sub> cassette (i.e., containing no E6/E7-targeted trans-acting Rz). Figure 8B is a bar graph plotting the relative HPV16 E6/E7 mRNA levels in cells transfected with SNIP<sub>AA</sub>Rz constructs expressing the indicated Rz. An empty SNIP<sub>AA</sub> cassette was used as control. Figure 8C is a bar graph plotting the relative HPV16 E6/E7 mRNA levels in cells transfected with SNIP<sub>AA</sub>Rz constructs expressing the indicated Rz. An empty SNIP<sub>AA</sub> cassette was used as control. mRz59 and mRz427 denote SNIP<sub>AA</sub> constructs containing catalytically inactive versions of Rz59 and Rz427, respectively. I Rz denotes an irrelevant SNIP<sub>AA</sub> construct containing a trans-acting Rz targeted to hepatitis B virus (Pan *et al.*, *RNA*, 7:610-621 (2001)), and pVAX1 denotes the vector control.

Figure 9A is a schematic of an Rz and Dz interacting with target RNA. The Rz and Dz cleavage sites are identified with an arrow. The numbering of Rz and Dz nucleotides that interact with the target RNA flanking the cleavage site are labeled as N 1 through 5 and Q 1 through 5. Figure 9B is a photograph of a gel used to measure the amount of target RNA cleavage for various mutant Rz and Dz. The specific mutations are described in Table 4.

Figure 10A is a bar graph plotting the relative level of HPV16 mRNA in cells containing the following nucleic acid one day after transfection (CMV promoter). Column 1: p1CLIP<sub>S</sub>/HPV16<sub>C+50-68+GUU</sub>; Column 2: p1CLIP<sub>AS</sub>/HPV16<sub>C+68-50+GUU</sub>; Column 3: pSIR/HPV16<sub>siC+50-68+GUU</sub>; Column 4: p1CLIP<sub>HP</sub>/HPV16<sub>C+50-68+GUU</sub>; Column 5:

pSIR<sub>HP</sub>/HPV16<sub>C+50-68+GUU</sub>; Column 6: p2CLIP<sub>HP</sub>/HPV16<sub>C+50-68+GUU</sub>; Column 7: p2CHOP<sub>HP</sub>/HPV16<sub>C+50-68+GUU</sub>; Column 8: pSNIP<sub>HP</sub>/HPV16<sub>C+50-68+GUU</sub>; Column 9: p2CLIP<sub>HR</sub>/HPV16<sub>C+50-68+GUU</sub>; Column 10: p2CHOP<sub>HR</sub>/HPV16<sub>C+50-68+GUU</sub>; Column 11: pSNIP<sub>HR</sub>/HPV16<sub>C+50-68+GUU</sub>; and Column 12: pSNIP (empty cassette). Figure 10B is a bar graph plotting the relative level of HPV16 mRNA in cells containing the following nucleic acid two days after transfection (U6 promoter). Column 1: p1CLIP<sub>S</sub>/HPV16<sub>C+50-68+GUU</sub>; Column 1: p1CLIP<sub>AS</sub>/HPV16<sub>C+50-68+GUU</sub>; Column 1: pSIR/HPV16<sub>siC+50-68+GUU</sub>; Column 1: p1CLIP<sub>HP</sub>/HPV16<sub>C+50-68+GUU</sub>; Column 1: pSIR<sub>HP</sub>/HPV16<sub>C+50-68+GUU</sub>; Column 1: p2CLIP<sub>HR</sub>/HPV16<sub>C+50-68+GUU</sub>; Column 1: p2CHOP<sub>HR</sub>/HPV16<sub>C+50-68+GUU</sub>; Column 1: pSNIP<sub>HR</sub>/HPV16<sub>C+50-68+GUU</sub>; Column 1: pSNIP cassette containing sRz59; and Column 1: pSNIP (empty cassette).

#### DETAILED DESCRIPTION

The invention provides methods and materials related to treating HPV infections and HPV-associated conditions. For example, the invention provides methods for treating mammals (e.g., humans) having cells (e.g., human cells) infected with HPV. The cells can be any type of cell such as skin cells or epithelial cells. In addition, the cells infected with HPV can be located anywhere within the mammal. For example, the HPV-infected cells can be located on the genital organ of the mammal. In some cases, the HPV-infected cells can form part of a genital wart or a cervical dysplasia. The HPV-infected cells also can contain integrated or non-integrated HPV nucleic acid. For example, the HPV-infected cells can contain HPV nucleic acid sequences that integrated into the genome of the cells. In some cases, the HPV-infected cells can lack HPV nucleic acid sequences in their genome. In these cases, HPV nucleic acid sequences can be maintained within the cells episomally. The HPV-infected cells also can exhibit HPV replication. For example, HPV-infected cells can be producing HPV particles.

Mammals having cells infected with any type of HPV can be treated as described herein. For example, humans having cells infected with HPV type 11, 16, 18, 31, 33, 35, 39, 45, 52, or 58 can be treated. In addition, mammals having cells infected with combinations of different HPV types can be treated as described herein. For example, a human having cells infected with both HPV16 and HPV18 can be treated.

As described herein, mammals having cells infected with HPV can be treated using nucleic acid molecules. The term "nucleic acid" as used herein encompasses both RNA and DNA, including cDNA, genomic DNA, and synthetic (e.g., chemically synthesized) DNA. The nucleic acid can be double-stranded or single-stranded. In addition, nucleic acid can be circular or linear. In some embodiments, the nucleic acid can be a plasmid. The nucleic acid can contain one or more restriction sites.

Typically, mammals having cells infected with HPV are treated using nucleic acid molecules that contain a sequence complementary to a nucleic acid sequence of the HPV. For example, antisense molecules, RNA molecules that induce RNA interference (e.g., double stranded RNA molecules, hairpin loop RNA molecules, siRNA molecules, or constructs designed to express such molecules), Rz, or Dz can be designed to have a sequence complementary to an HPV sequence. Such nucleic acid molecules can be complementary to any HPV sequence including, without limitation, non-coding HPV nucleic acid sequences (e.g., poly(A) signal sequences and regulatory sequences such as the upstream regulatory region) and HPV polypeptide-encoding nucleic acid sequences (e.g., E1, E2, E3, E4, E5, E6, E7, L1, and L2 polypeptide-encoding sequences). In some cases, the nucleic acid molecules are designed based on accessible HPV RNA target sites. Such target sites can be identified using any method such as the library selection methods described herein (e.g., LS1, LS2, and LS3). In addition, any method can be used to make nucleic acid molecules such as antisense molecules, RNA molecules that induce RNA interference (e.g., double stranded RNA molecules, hairpin loop RNA molecules, siRNA molecules, or constructs designed to express such molecules), Rz, or Dz having a sequence complementary to an HPV sequence. For example, the methods and materials described in International Patent Application No. PCT/US01/46178 and U.S. Provisional Patent Application No. 60/449,066 can be used.

The term "complementary" as used herein with reference to two nucleic acid sequences (e.g., a Rz or Dz recognition sequence and an HPV mRNA target sequence; or an antisense oligonucleotide sequence and an HPV mRNA target sequence) means the two nucleic acid sequences are 100 percent complementary. The complementary sequences can be part of larger nucleic acid molecules having sequences that are not complementary. The length of the complementary sequence can be any length greater

than 3 nucleotides (e.g., 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more nucleotides). For example, a 60-nucleotide Dz can have a 10-nucleotide sequence complementary to an HPV mRNA sequence. Typically, complementary sequences range in length from about 4 nucleotides to about 20  
5 nucleotides (e.g., from about 5 nucleotides to about 18 nucleotides, or from about 6 nucleotides to about 15 nucleotides).

Any method can be used to administer a nucleic acid molecule to a mammal containing cells infected with HPV. For example, administration can be topical (e.g., transdermal, ophthalmic, or intranasal); pulmonary (e.g., by inhalation or insufflation of  
10 powders or aerosols); oral; or parenteral (e.g., by subcutaneous, intrathecal, intraventricular, intramuscular, or intraperitoneal injection, or by intravenous drip). Administration can be rapid (e.g., by injection), or can occur over a period of time (e.g., by slow infusion or administration of slow release formulations).

Typically, when treating a mammal having cells infected with HPV, the nucleic  
15 acid molecules are administered topically. For example, nucleic acid molecules formulated with or without liposomes can be topically applied directly to HPV<sup>+</sup> precancerous cervical dysplasia tissue or HPV lesions such as genital warts. For topical administration, a nucleic acid molecule can be formulated into compositions such as sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such  
20 as alcohols, or solutions in liquid or solid oil bases. Such solutions also can contain buffers, diluents, and other suitable additives. Compositions for topical administration can be formulated in the form of transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids, and powders. Coated condoms, gloves, and the like also can be used. Conventional pharmaceutical carriers, aqueous, powder or oily bases,  
25 thickeners, and the like can be added.

For oral administration, a nucleic acid molecule can be formulated into compositions such as powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Such compositions also can incorporate thickeners, flavoring agents, diluents, emulsifiers, dispersing aids, or binders.

30 For parenteral, intrathecal, or intraventricular administration, a nucleic acid molecule can be formulated into compositions such as sterile aqueous solutions, which

also can contain buffers, diluents, and other suitable additives (e.g., penetration enhancers, carrier compounds, and other pharmaceutically acceptable carriers).

In addition, nucleic acid molecules can be administered to a mammal containing HPV-infected cells using biologic or abiologic means as described in, for example, U.S.

- 5 Patent No. 6,271,359. Abiologic delivery can be accomplished by a variety of methods including, without limitation, (1) loading liposomes with a nucleic acid molecule provided herein and (2) complexing a nucleic acid molecule with lipids or liposomes to form nucleic acid-lipid or nucleic acid-liposome complexes. The liposome can be composed of cationic and neutral lipids commonly used to transfect cells *in vitro*.
- 10 Cationic lipids can complex (e.g., charge-associate) with negatively charged nucleic acids to form liposomes. Examples of cationic liposomes include, without limitation, lipofectin, lipofectamine, lipofectace, and DOTAP. Procedures for forming liposomes are well known in the art. Liposome compositions can be formed, for example, from phosphatidylcholine, dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine,
- 15 dimyristoyl phosphatidylglycerol, or dioleoyl phosphatidylethanolamine. Numerous lipophilic agents are commercially available, including Lipofectin<sup>®</sup> (Invitrogen/Life Technologies, Carlsbad, CA) and Effectene<sup>™</sup> (Qiagen, Valencia, CA). In addition, systemic delivery methods can be optimized using commercially available cationic lipids such as DDAB or DOTAP, each of which can be mixed with a neutral lipid such as
- 20 DOPE or cholesterol. In some cases, liposomes such as those described by Templeton *et al.* (*Nature Biotechnology*, 15:647-652 (1997)) can be used. In other embodiments, polycations such as polyethyleneimine can be used to achieve delivery *in vivo* and *ex vivo* (Boletta *et al.*, *J. Am Soc. Nephrol.* 7: 1728 (1996)). Additional information regarding the use of liposomes to deliver nucleic acids can be found in U.S. Patent No. 6,271,359.

- 25 Biologic delivery can be accomplished by a variety of methods including, without limitation, the use of viral vectors. For example, viral vectors (e.g., adenovirus and herpesvirus vectors) can be used to deliver nucleic acid molecules to skin cells and cervical cells. Standard molecular biology techniques can be used to introduce one or more of the nucleic acid molecules provided herein into one of the many different viral
- 30 vectors previously developed to deliver nucleic acid to cells. These resulting viral vectors can be used to deliver the one or more nucleic acid molecules to cells by, for example,

infection.

Nucleic acid molecules having a sequence complementary to an HPV sequence can be formulated in a pharmaceutically acceptable carrier or diluent. A

“pharmaceutically acceptable carrier” (also referred to herein as an “excipient”) is a

5 pharmaceutically acceptable solvent, suspending agent, or any other pharmacologically inert vehicle. Pharmaceutically acceptable carriers can be liquid or solid, and can be selected with the planned manner of administration in mind so as to provide for the desired bulk, consistency, and other pertinent transport and chemical properties. Typical pharmaceutically acceptable carriers include, by way of example and not limitation:

10 water; saline solution; binding agents (e.g., polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose and other sugars, gelatin, or calcium sulfate); lubricants (e.g., starch, polyethylene glycol, or sodium acetate); disintegrates (e.g., starch or sodium starch glycolate); and wetting agents (e.g., sodium lauryl sulfate).

In addition, nucleic acid molecules having a sequence complementary to an HPV

15 sequence can be formulated into compositions containing the nucleic acid molecule admixed, encapsulated, conjugated, or otherwise associated with other molecules, molecular structures, or mixtures of nucleic acids. For example, a composition containing one or more nucleic acid molecules having a sequence complementary to an HPV sequence can contain other therapeutic agents such as anti-inflammatory drugs (e.g.,

20 nonsteroidal anti-inflammatory drugs and corticosteroids) and antiviral drugs (e.g., ribivirin, vidarabine, acyclovir, and ganciclovir). In some embodiments, a composition can contain one or more nucleic acid molecules having a sequence complementary to an HPV sequence in combination with a keratolytic agent. Keratolytic agents are agents that separate or loosen the horny layer of the epidermis. An example of a keratolytic agent

25 includes, without limitation, salicylic acid. Other examples are provided in U.S. Patent No. 5,543,417. Keratolytic agents can be used in an amount effective to enhance the penetration of nucleic acid molecules, for example, into tissues such as skin. For example, a keratolytic agent can be used in an amount that allows a nucleic acid molecule applied to a genital wart to penetrate throughout the wart.

30 In addition, nucleic acid molecules can be administered in amounts and for periods of time that vary depending upon the age, weight, and condition of the mammal,

the nature of the particular HPV infection and its severity, the mammal's overall condition, and the particular compound delivered. Typically, a nucleic acid molecule can be administered in an inhibitory amount such as an amount that inhibits expression of an HPV polypeptide (e.g., E6 or E7 polypeptide). The inhibition can be at any level of inhibition. For example, HPV polypeptide expression can be inhibited by 5, 10, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or more percent. In some cases, the inhibition can result in an absence of detectable HPV polypeptide expression. Dosing can be based on the severity and responsiveness of the disease state to be treated, with, for example, the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Routine methods can be used to determine optimum dosages, dosing methodologies, and repetition rates. Optimum dosages can vary depending on the relative potency of individual nucleic acid molecules, and can generally be estimated based on EC<sub>50</sub> values found to be effective in *in vitro* and/or *in vivo* models. Typically, a dosage is from about 0.01 µg to about 100 g per kg of body weight, and can be given once or more daily, weekly, or even less often. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state.

As described herein, nucleic acid molecules having a sequence complementary to an HPV sequence can be administered to a mammal under conditions wherein the number of cells infected with the HPV is reduced. This reduction can be any detectable level of reduction. For example, the number of HPV-infected cells can be reduced by 5, 10, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or more percent. In some cases, the reduction can result in an absence of detectable HPV-infected cells.

The presence or absence of HPV-infected cells can be determined using any method. For example, Southern blots, PCR, Northern blots, RT-PCR, immunostaining, ELISAs, and radioimmunoassays can be used to detect HPV DNA, RNA, or polypeptides in a cellular sample. In addition, any method can be used to determine the number of HPV-infected cells within a mammal. For example, standard molecular biology techniques such as *in situ* hybridization and immunostaining can be used to determine the number of HPV-infected cells. Likewise, any method can be used to determine whether or not the number of HPV-infected cells within a mammal was reduced. For example,

tissues can be examined macroscopically, microscopically, immunologically, biochemically, or molecularly before and after treatment to determine whether the number of HPV-infected cells was reduced. Typically, a macroscopically observable size reduction or disappearance of a genital wart (known to contain HPV-infected cells)

5 following treatment indicates that the number of HPV-infected cells was reduced.

The invention also provides isolated nucleic acids. The term "isolated" as used herein with reference to nucleic acid refers to a naturally-occurring nucleic acid that is not immediately contiguous with both of the sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally-occurring genome of  
10 the organism from which it is derived. For example, an isolated nucleic acid can be, without limitation, a recombinant DNA molecule of any length, provided one of the nucleic acid sequences normally found immediately flanking that recombinant DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid includes, without limitation, a recombinant DNA that exists as a separate molecule  
15 (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, lentivirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid can include a recombinant  
20 DNA molecule that is part of a hybrid or fusion nucleic acid sequence.

The term "isolated" as used herein with reference to nucleic acid also includes any non-naturally-occurring nucleic acid since non-naturally-occurring nucleic acid sequences are not found in nature and do not have immediately contiguous sequences in a naturally-occurring genome. For example, non-naturally-occurring nucleic acid such as an  
25 engineered nucleic acid is considered to be isolated nucleic acid. Engineered nucleic acid can be made using common molecular cloning or chemical nucleic acid synthesis techniques. Isolated non-naturally-occurring nucleic acid can be independent of other sequences, or incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, lentivirus, adenovirus, or herpes virus), or the genomic DNA of a  
30 prokaryote or eukaryote.

It will be apparent to those of skill in the art that a nucleic acid existing among



hundreds to millions of other nucleic acid molecules within, for example, cDNA or genomic libraries, or gel slices containing a genomic DNA restriction digest is not to be considered an isolated nucleic acid.

The isolated nucleic acids provided herein can be RNA or DNA and can contain one or more catalytic core sequences. The term "catalytic core sequence" as used herein refers to a sequence that is primarily responsible for the catalytic activity of a Dz or Rz, as opposed to target recognition. Examples of catalytic core sequences include, without limitation, SEQ ID NO:51 and SEQ ID NO:68. Other catalytic core sequences are described elsewhere (Santoro and Joyce, *Proc. Natl. Acad. Sci.*, 94(9):4262-6 (1997); Feldman and Sen, *Mol. Biol.*, 313(2):283-94 (2001); and Okumoto *et al.*, *Biochemistry*, 42(7):2158-65 (2003)).

In addition to one or more catalytic core sequences, an isolated nucleic acid provided herein can contain one or more recognition sequences. Typically, a recognition sequence is a sequence complementary to a target sequence such as a target HPV mRNA sequence. In some embodiments, the isolated nucleic acid can contain a 5' recognition sequence followed by a catalytic core sequence followed by a 3' recognition sequence. The recognition sequences can be complementary to any HPV sequence such as an HPV E6/E7 mRNA sequence. The isolated nucleic acids can be designed such that they cleave a cleavage site of an HPV mRNA. Examples of HPV mRNA cleavage sites accessible to Dz and Rz cleavage can be found in Tables 1a, 1b, and 1c.

The invention also provides isolated, single-stranded nucleic acid molecules. Such nucleic acid molecules can consist of between 5 and 40 nucleotides (e.g., between 6 and 35 nucleotides, between 7 and 30 nucleotides, between 8 and 25 nucleotides, or between 8 and 20 nucleotides). In addition, these nucleic acid molecules can have a sequence complementary to at least five consecutive nucleotides (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more consecutive nucleotides) of an HPV mRNA sequence. For example, an isolated, single-stranded nucleic acid molecule provided herein can have a sequence complementary to at least five consecutive nucleotides of the sequences set forth in Tables 1a, 1b, and 1c.

In addition, the invention provides isolated, double-stranded RNA molecules. Typically, one strand of such RNA molecules contains a sequence complementary to at

least five consecutive nucleotides (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more consecutive nucleotides) of an HPV mRNA sequence. For example, an isolated, double-stranded RNA molecule provided herein can have a strand having a sequence complementary to at least five consecutive nucleotides of  
5 the sequences set forth in Tables 1a, 1b, and 1c.

In some embodiments, the isolated, double-stranded RNA molecules can be designed to be siRNA molecules. Typically, siRNA molecules are short, double-stranded RNA molecules. In addition, siRNA molecules can be any length (e.g., 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, or more nucleotides in length). For example, siRNA molecules can  
10 be 15 to 30 nucleotides in length (e.g., 18 to 25, 20 to 24, or 20 to 21 nucleotides in length). In some embodiments, the selected target sequences from HPV RNA can be used to design siRNA molecules. For example, the antisense molecules provided herein can be synthesized as double-stranded RNA molecules. In addition, the length of the siRNA molecules can be identical to the length of the antisense molecules provided  
15 herein or the length can be increased or decreased (e.g., the length can be increased or decreased such that the siRNA molecules are 20 to 21 nucleotides in length). Any method can be used to deliver siRNA molecules to cells. For example, siRNA molecules can be applied to cells using the methods described herein for the antisense molecules. Alternatively, a sequence encoding an siRNA molecule can be incorporated into an  
20 expression construct that can be introduced into cells so that siRNA molecules are expressed.

The invention also provides methods and materials for obtaining single-stranded DNA (e.g., Dz). Such methods and materials can be used to make libraries of single-stranded Dz. For example, an amplification reaction (e.g., PCR reaction) can be  
25 performed using a labeled primer (e.g., a biotin-labeled primer) such that one strand of the resulting double-stranded nucleic acid contains the label. Once amplified, the amplification products can be incubated with a binding agent (e.g., streptavidin) that interacts with the label, allowing the labeled strand to be separated from unlabeled strands. For example, streptavidin-coated resin can be used to remove biotin-containing  
30 strands from strands lacking biotin. Typically, the amplification reactions can be designed using primers such that the strand containing Dz sequences (e.g., a library of

different Dz sequences) is unlabeled and the complement strand is labeled, for example, with biotin. In this case, the Dz strands can be obtained by collecting material that does not bind to a binding agent resin, for example, streptavidin-coated beads.

Single-stranded DNA also can be obtained using amplification reactions with one  
5 or more primers containing one or more ribose nucleotides. The ribose nucleotide or nucleotides can be located anywhere along the primer. Typically, at least one ribose nucleotide is located at or near the 3' end of the primer. Once amplified, the resulting double-stranded nucleic acid can have one strand that contains one or more ribose nucleotides. Treatment of this resulting product with a base hydrolysis agent such as  
10 sodium hydroxide can result in cleavage of the strand containing a ribose nucleotide at the ribose nucleotide. Typically, the amplification products are treated with NaOH with a final NaOH concentration of, for example, about 0.1, 0.25, 0.5, or 0.75 M. In addition, once the sodium hydroxide is added, the mixture can be heated (e.g., to about 80°C, 85°C, 90°C, or 95°C) for several minutes (e.g., about 5, 10, 15 minutes) and then cooled (e.g.,  
15 placed on ice).

Once the strand containing at least one ribose nucleotide is cleaved, the mixture can be neutralized by adding, for example, HCl with a final HCl concentration of, for example, about 0.1, 0.25, 0.5, or 0.75 M. The two strands can be separated based on size. For example, PAGE (e.g., an 8 percent denaturing polyacrylamide gel) can be used to  
20 separate the shorter cleaved strand from a longer uncleaved strand. The band corresponding to the strand containing Dz sequences can be isolated, and the single-stranded Dz eluted.

Single-stranded DNA also can be obtained using amplification reactions with one or more primers containing a carbon spacer. The carbon spacer can be located anywhere  
25 along the primer. During amplification reactions, the polymerase typically is unable to extend beyond the carbon spacer, resulting in one strand being longer than the other. The differentially sized strands can be separated, and the strand containing Dz sequences isolated.

The term "carbon spacer" as used herein refers to a non-pentose molecular  
30 structure that contains one or more carbon-carbon backbone bonds. Typically, a polymerase (e.g., Taq polymerase or a reverse transcriptase) can not polymerize nucleic

acid through the carbon spacer region of a template containing a carbon spacer. A carbon spacer can contain repeating oxygen-carbon-carbon units. For example, a carbon spacer can be 2, 3, 4, 5, 6, 7, 8, 9, 10, or more oxygen-carbon-carbon units. In addition, a carbon spacer can contain a phosphate group. For example, six oxygen-carbon-carbon units can be followed by a phosphate group (Figure 3). Typically, the primer will contain a 5' nucleic acid sequence following by one or more oxygen-carbon-carbon units followed by a phosphate group followed by a 3' nucleic acid sequence. Such primers can contain any nucleic acid sequence.

In some embodiments, the invention provides nucleic acid molecules having a nucleic acid sequence (represented as  $(N)_{n1}$ , where N is any nucleotide, and  $n1$  is an integer greater than 0) followed by a carbon spacer (e.g., an oxygen-carbon-carbon unit represented as  $(\text{oxygen-carbon-carbon})_{n2}$ , where  $n2$  is an integer greater than 0) followed by another nucleic acid sequence (represented as  $(N)_{n3}$ , where N is any nucleotide, and  $n3$  is integer greater than 0). The nucleic acid sequences represented as  $(N)_{n1}$  and  $(N)_{n3}$  can have any sequence. For example,  $(N)_{n1}$  can be 5'-ATGG-3', and  $(N)_{n3}$  can be 5'-CTGAC-3'.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

20

## EXAMPLES

### Example 1 - Library selection of target sites

Full-length HPV11<sub>E6/E7</sub>, HPV16<sub>E6/E7</sub>, and HPV18<sub>E6/E7</sub> RNA was transcribed and subjected to library selection using a random pool of oligonucleotides (LS1), a pool of active Rz (LS2) with random flanking sequences, or a pool of active Dz (LS3) with random flanking sequences. Briefly, the LS1 library selection protocol involved using a random oligonucleotide library SELEX procedure (5 rounds) according to Pan *et al.* (RNA, 7:610-21 (2001)). The LS2 library selection was performed using a random library of active Rz (Pan *et al.*, Mol. Therapy, 7:128-138 (2003) and International Patent Application No. PCT/US01/46178). Briefly, the library was allowed to bind to target RNA (e.g., HPV11<sub>E6/E7</sub>, HPV16<sub>E6/E7</sub>, or HPV18<sub>E6/E7</sub> RNA) under inactive conditions, and the bound species were reamplified. A second round of binding and amplification was

performed at a lower target RNA:Rz ratio to increase specificity. The selected pool was then transcribed into active Rz that were incubated with full-length 5'-<sup>32</sup>P-HPV11<sub>E6/E7</sub>, HPV16<sub>E6/E7</sub>, or HPV18<sub>E6/E7</sub> labeled target RNA. Cleavage products were separated on sequencing gels, and sites of cleavage were directly identified by comparison with G-, T-,  
5 and base hydrolysis ladders.

The LS3 library selection was performed using a random library of active Dz (Figure 1; International Patent Application No. PCT/US01/46178). Briefly, a library of single-stranded Dz was prepared and allowed to bind to target RNA (e.g., HPV11<sub>E6/E7</sub>, HPV16<sub>E6/E7</sub>, or HPV18<sub>E6/E7</sub> RNA) under inactive conditions (85°C and no Mg<sup>++</sup>), and the  
10 bound species were obtained by nondenaturing PAGE and reamplified. A second round of binding and amplification was performed at a lower target RNA:Dz ratio to increase specificity. Single-stranded Dz were obtained from the selected pool and incubated with full-length 5'-<sup>32</sup>P-HPV11<sub>E6/E7</sub>, HPV16<sub>E6/E7</sub>, or HPV18<sub>E6/E7</sub> labeled target RNA under active conditions (37°C and 25 mM MgCl). Cleavage products were separated on  
15 sequencing gels, and sites of cleavage were directly identified by comparison with G-, T-, and base hydrolysis ladders. Both one and two rounds of selection using the LS3 protocol resulted in detectable cleavage. These results indicate that accessible cleavage sites can be identified using one round of selection with the LS3 protocol, although sites are more readily apparent after two rounds of selection.

20 During the LS3 protocol, the single-stranded Dz were obtained using a PCR reaction where a biotin-labeled primer was used to prime the strand lacking the Dz sequence. A streptavidin-containing resin was then used to separate the biotin-labeled strand from the Dz-containing strand. Alternatively, ribose-containing primers (e.g., DNA primers where the base at the 3' end is a ribose) were used to perform amplification  
25 reactions that resulted in one strand containing an RNA nucleotide (Figure 2). Sodium hydroxide (0.5 M final concentration) was added to the amplification reaction product to cleave the strand containing the ribose nucleotide between the ribose and 2-deoxyribose nucleotides. After adding sodium hydroxide, the mixture was incubated at 95°C for 10 minutes and then placed on ice. The resulting mixture was neutralized with HCl, and the  
30 two strands separated based on size using an 8 percent denaturing polyacrylamide gel. The band corresponding to the strand containing the Dz sequence was isolated, and the

single-stranded Dz were eluted. A third method involved using a primer that contained a carbon spacer. The carbon spacer contained repeating oxygen-carbon-carbon units followed by a phosphate group (Figure 3). During amplification reactions, the polymerase is unable to extend beyond the carbon spacer, resulting in one strand being longer than the other. The differentially sized strands were then separated, and the strand containing the Dz sequences isolated.

A number of sites were identified with each protocol (Tables 1a and 1b). In addition, several sites were identified using both the LS1 and LS2 protocols (e.g., a site surrounding nucleotide position 407 of HPV11<sub>E6/E7</sub> RNA, which corresponds to nucleotide position 505 in Genbank<sup>®</sup> accession number M14119; Table 1b). HPV target sequences were also selected without a library selection protocol. For example, several sites within E5 close to a common poly(A) signal used for the majority of HPV transcripts were selected (Table 1c).

Table 1a. HPV target sequences identified using library selection protocols and used to make Rz.

Target mRNA	Genbank <sup>®</sup> Accession Number	Rz	Cleavage Site	Library Selection Protocol	Target Sequence
HPV16 E6/E7	K02718	31	110	LS1	UGCAAUGUUUCAGGACC (SEQ ID NO:4)
		59	138	LS2	CCCAGAAAGUUACCACA (SEQ ID NO:5)
		68	147	LS2	UUACCACAGUUAUGCAC (SEQ ID NO:6)
		150	229	LS2	GACGUGAGGUUAUGAC (SEQ ID NO:7)
		187	266	LS2	CAUAGUAUAUAGAGAUG (SEQ ID NO:8)
		251	330	LS2	AUUAGUGAGUAUAGACA (SEQ ID NO:9)
		275	354	LS2	UAUAGUUUGUAUGGAAC (SEQ ID NO:10)
		358	437	LS1	GCCACUGUGUCCUGAAG (SEQ ID NO:11)
		415	494	LS2	UAUAAGGGGUCGGUGGA (SEQ ID NO:12)
		427	506	LS1/LS2	GUGGACCGGUCGAUGUA

					(SEQ ID NO:13)
HPV11 E6/E7	M14119	23	121	LS1	AAAGAUGCCUCCACGUC (SEQ ID NO:14)
		162	260	LS2	ACCUAAAGGUUGUGUGG (SEQ ID NO:15)
		244	342	LS2	UAGACACUUUAAUUAUG (SEQ ID NO:16)
		345	443	LS1	UGUGUGAAAUAGAAAAA (SEQ ID NO:17)
		409	507	LS1/LS2	GUGGAAGGGUCGUUGCU (SEQ ID NO:18)
		449	547	LS2	GAAGACUUGUUACCCUA (SEQ ID NO:19)
		499	597	LS2	CCUGUAGGGUUACAUUG (SEQ ID NO:20)
		529	627	LS2	GAAGACAGCUCAGAAGA (SEQ ID NO:21)
		593	691	LS2	AUUACCAAUACUGACC (SEQ ID NO:22)
HPV18 E6/E7	X05015	19	123	LS1	CUUUGAGGAUCCAACAC (SEQ ID NO:23)
		340	444	LS1	ACCGUUGAAUCCAGCAG (SEQ ID NO:24)
		403	507	LS1	UGGGCACUAUAGAGGCC (SEQ ID NO:25)

The underlined nucleotides represent Rz cleavage sites.

Table 1b. HPV target sequences identified using library selection protocols and used to make Dz.

Target mRNA	Genbank <sup>®</sup> Accession Number	Dz	Cleavage Site	Library Selection Protocol	Target Sequence
HPV16 E6/E7	K02718	57	136	LS2	CCCAGAAAGTTACCACA (SEQ ID NO:26)
		66	145	LS2	TTACCACAGTTATGCAC (SEQ ID NO:27)
		148	227	LS2	GACGTGAGGTATATGAC (SEQ ID NO:28)
		185	264	LS2	CATAGTATATAGAGATG (SEQ ID NO:29)
		249	328	LS2	ATTAGTGAGTATAGACA (SEQ ID NO:30)
		273	352	LS2	TATAGTTTGTATGGAAC (SEQ ID NO:31)

		413	492	LS2	TATAAGGGGTCGGTGGA (SEQ ID NO:32)
		425	504	LS1/LS2	GTGGACCGGTCGATGTA (SEQ ID NO:33)
		589	668	LS3	GGAGGAGGATGAAATAG (SEQ ID NO:34)
HPV11 E6/E7	M14119	27	125	LS1	GCCTCCACGTCTGCAAC (SEQ ID NO:35)
		160	258	LS2	ACCTAAAGGTTGTGTGG (SEQ ID NO:36)
		231	329	LS2	ATTAACCAATATAGACA (SEQ ID NO:37)
		323	421	LS2	TTACCTGTGTCACAAGC (SEQ ID NO:38)
		338	436	LS1	GCCGTTGTGTGAAATAG (SEQ ID NO:39)
		407	505	LS1/LS2	GTGGAAGGGTCGTTGCT (SEQ ID NO:40)
		447	545	LS2	GAAGACTTGTTACCCTA (SEQ ID NO:41)
		526	624	LS2	AGAAGACAGCTCAGAAG (SEQ ID NO:42)
		591	689	LS2	ATTACCAAATACTGACC (SEQ ID NO:43)
HPV18 E6/E7	X05015	17	121	LS1	CTTTGAGGATCCAACAC (SEQ ID NO:44)
		338	442	LS1	ACCGTTGAATCCAGCAG (SEQ ID NO:45)
		401	505	LS1	TGGGCACTATAGAGGCC (SEQ ID NO:46)

The bold nucleotides represent Dz cleavage sites.

Table 1c. HPV target sequences within E5 close to a common poly(A) signal used for the majority of HPV transcripts.

Target mRNA	Genbank <sup>®</sup> Accession Number	Dz	Cleavage Site	Target Sequence
HPV16 E6/E7	K02718	AA3916	3916	GTGCTTTTGTGTGTCTG (SEQ ID NO:47)
		AA4015	4015	GCCTCTGCGTTTAGGTG (SEQ ID NO:48)

5 The bold nucleotides represent Dz cleavage sites.



### Example 2 – Design of ASO, Rz, and Dz

ASO, Rz, and Dz were designed to target sites identified using the library selection protocols. For example, ASO407<sub>(HPV11-E6/E7)</sub> was designed to bind to the region surrounding 409 (Table 1a) and has the following sequence: 5'-AGCAACGACC-  
 5 CTTCCAC-3' (SEQ ID NO:49). A protected version of ASO407<sub>(HPV11-E6/E7)</sub>, ASO407<sub>p(HPV11-E6/E7)</sub>, was designed to have the following sequence: 5'-AGCAAC-GACCCTTCCAC-T\*-3' (SEQ ID NO:50). The "T\*" indicates that the thymidine is inverted (Santiago *et al.*, *Nat. Med.*, 5(11):1264-69 (1999)). As described below, this modification significantly protected the ASO and Dz against degradation in  
 10 medium/serum and in cells.

Five Rz, Rz23<sub>(HPV11-E6/E7)</sub>, Rz162<sub>(HPV11-E6/E7)</sub>, Rz244<sub>(HPV11-E6/E7)</sub>, Rz345<sub>(HPV11-E6/E7)</sub>, and Rz409<sub>(HPV11-E6/E7)</sub>, were designed to have a catalytic core sequence (e.g., 5'-CUGAUGAGUCCGUGAGGACGAAA-3'; SEQ ID NO:51) flanked by sequences complementary to the target site sequences. For example, Rz59<sub>(HPV16-E6/E7)</sub>, designed to  
 15 cut cleavage site 138 of HPV16 E6/E7 RNA, can have the following sequence: 5'-UGUGGUCUGAUGAGUCCGUGAGGACGAAACUUUCUGGG-3' (SEQ ID NO:52) where the underlined sequence represents the catalytic core sequence. The Rz cut sites were chosen within the accessible regions according to Rz specificity considerations (e.g., NUC triplet for Rz).

20 The template sequence for each Rz was placed in a SNIP<sub>AA</sub> expression cassette (Figure 5A) either as single internal trans-acting Rz (ITRz) or as double ITRz (dITRz). Briefly, this cassette consists of two triple Rz (Zhang *et al.*, *Exp. Cell Res.*, 273:73-84 (2002)). Each of the triple ribozymes, in turn, consists of two cis-acting ribozymes flanking the ITRz or dITRz. The two cis-acting Rz cleave the primary transcript,  
 25 liberating the ITRz or dITRz with minimal extraneous flanking sequences. Use of this cassette provides an additional advantage in that the liberated ITRz or dITRz is distributed between nucleus (5-20%) and cytoplasm (Pan *et al.*, *RNA*, 7:610-21 (2001); Benedict *et al.*, *Carcinogenesis*, 19:1223-30 (1998); Ren *et al.*, *Gene Therapy and Mol. Biol.*, 3:1-13 (1998); and Crone *et al.*, *Hepatology*, 29:1114-23 (1999)).

30 The ITRz or dITRz liberated from the CLIP portion of the SNIP<sub>AA</sub> cassette contained a flanking sequence at the 5' end (5'-AGCUCGACCUCAGAUCU-3'; SEQ ID

NO:53) and at the 3' end (5'-CAAUUGAAAAAAAAAAAAAAAAAAAAAAAAAA-  
AAAGUC-3'; SEQ ID NO:54). The ITRz or dITRz liberated from the CHOP portion of  
the SNIP<sub>AA</sub> cassette contained a flanking sequence at the 5' end (5'-GGUCCAG-  
GAUCC-3'; SEQ ID NO:55) and at the 3' end (5'-GAAUUCAAAAAAAAAAAAAAAA-  
5 AAAAAAAAAAAAAAAAAAGUC-3'; SEQ ID NO:56). The "catalytic core" of the  
various ITRz and dITRz Rz consisted of the following sequence: 5'-CUGAUGA-  
GUCCGUGAGGACGAAA-3' (SEQ ID NO:51). The flanking sequences were as  
follows for the indicated ITRz:

Rz162: ---CCACAC-"Catalytic Core"-CCUUUAGGU--- (SEQ ID NO:57)  
10 Rz244: ---CAUAAU-"Catalytic Core"-AAGUGUCUA--- (SEQ ID NO:58)  
Rz409: ---AGCAAC-"Catalytic Core"-CCCUUCCAC--- (SEQ ID NO:59)

The flanking sequences were as follows for the indicated dITRz:

Rz409/162: ---AGCAAC-"Catalytic Core"-CCCUUCCAC-CCACAC-  
"Catalytic Core"-AAGTGTCTA--- (SEQ ID NO:60)  
15 Rz409/244: ---AGCAAC-"Catalytic Core"-CCCUUCCAC-CAUAAU-  
"Catalytic Core"-AAGUGUCUA--- (SEQ ID NO:61)  
Rz409/409: ---AGCAAC-"Catalytic Core"-CCCUUCCAC-AGCAAC-  
"Catalytic Core"-CCCUUCCAC--- (SEQ ID NO:62)

In addition, the ITRz409 from the CLIP portion of the SNIP<sub>AA</sub> cassette contained the  
20 following sequence: 5'- AGCUCGACCUCAGAUCUAGCAACCUGAUGAGUCC-  
GUGAGGACGAACCCUUCCACCAUUGAAAAAAAAAAAAAAAAAAAAAAAAA  
AAAAAAGUC-3' (SEQ ID NO:63). The HPV11-specific flanking sequences are  
underlined. The dITRz409/409 from the CLIP portion of the SNIP<sub>AA</sub> cassette contained  
the following sequence: 5'-AGCUCGACCUCAGAUCUAGCAACCUGAUGAGUC-  
25 CGUGAGGACGAACCCUUCCACAGCAACCUGAUGAGUCCGUGAGGACGAAA  
CCCUUCCACCAUUGAAAAAAAAAAAAAAAAAAAAAAAAAAGUC-3'  
(SEQ ID NO:64). Again, the HPV11-specific flanking sequences are underlined.

Dz27<sub>(HPV11-E6/E7)</sub>, Dz338<sub>(HPV11-E6/E7)</sub>, and Dz407<sub>(HPV11-E6/E7)</sub> were designed to cleave  
cut sites identified with the LS1 protocol, since Dz targeted to sites identified with the  
30 LS2 protocol can exhibit poor activity. The Dz cut sites were chosen within the  
accessible regions according to Dz specificity considerations (e.g., an R-Y doublet for

Dz). Dz27<sub>(HPV11-E6/E7)</sub>, which targets cleavage site 125 of HPV11 E6/E7 RNA, was designed to have the following sequence: 5'-GTTGCAGAGGCTAGCTACAACGAG-TGGAGGC-3' (SEQ ID NO:65). Dz338<sub>(HPV11-E6/E7)</sub>, which targets cleavage site 436 of HPV11 E6/E7 RNA, was designed to have the following sequence: 5'-CTATTTCA-  
 5 GGCTAGCTACAACGAACAACGGC-3' (SEQ ID NO:66). Dz407<sub>(HPV11-E6/E7)</sub>, which targets cleavage site 505 of HPV11 E6/E7 RNA, was designed to have the following sequence: 5'-AGCAACGAGGCTAGCTACAACGACCTTCCAC-3' (SEQ ID NO:67). In each case, the catalytic core of the Dz is underlined (5'-GGCTAGCTACAACGA-3'; SEQ ID NO:68).

10 A catalytically inactive Dz targeted to 407 (Dz407m<sub>(HPV11-E6/E7)</sub>) was designed and used as a control. Dz407m<sub>(HPV11-E6/E7)</sub> has the following sequence: 5'-AGCAACG-AGCCTAGCTACTACGACCTTCCAC-3' (SEQ ID NO:69). The nucleotide substitutions, which eliminate Dz catalytic activity, are shown in bold. DzAR2000 and a catalytically inactive version of DzAR2000, DzAR2000m, were designed to target an  
 15 androgen receptor RNA sequence and used as controls. DzAR2000 has the following sequence: 5'-TCCGAAGAGGCTAGCTACAACGAGACAAGAT-3' (SEQ ID NO:70). DzAR2000m has the following sequence: 5'-TCCGAAGAGCCTAGCTACTACGAG-ACAAGAT-3' (SEQ ID NO:71).

### 20 Example 3 - Analysis of *in vitro* cleavage activity

Once designed, the Rz and Dz were tested *in vitro* for activity against E6/E7 target RNA under conditions as described elsewhere (Pan *et al.*, *RNA*, 7:610-21 (2001)). The Rz were obtained by synthesizing overlapping oligodeoxynucleotides, which encoded the ITRz sequence downstream of a 5' T7 promoter. The ITRz (identical to those liberated  
 25 from the CHOP portion of the SNIP cassette) were then transcribed *in vitro* and gel purified. The Dz were obtained from DNagency (Malvern, PA).

Rz (Rz23<sub>(HPV11-E6/E7)</sub>, Rz409<sub>(HPV11-E6/E7)</sub>, and Rz345<sub>(HPV11-E6/E7)</sub>) were designed based on identified sites, transcribed *in vitro*, and gel purified. Rz (at 50 nM) were mixed with full length <sup>32</sup>P-labeled HPV11 E6 target mRNA (at 200 nM) and incubated for 30  
 30 minutes or 2 hours at 37°C, and the cut products were then examined by PAGE and autoradiography. Rz409<sub>(HPV11-E6/E7)</sub> and Rz23<sub>(HPV11-E6/E7)</sub> were the most active Rz (Figure

4A). The HPV16-targeted Rz (targeted to the homologous region in HPV 16 E6/E7, nucleotide position 427) exhibited no activity against the HPV11 E6 target mRNA. This HPV16 Rz and the HPV11 E6 target mRNA target differ in the 2<sup>nd</sup>, 5<sup>th</sup>, and 6<sup>th</sup> nucleotides downstream from the cutting site, and in the 5<sup>th</sup> and 6<sup>th</sup> nucleotides upstream.

- 5 The activity of the HPV16-targeted Rz against an HPV16 E6/E7 target mRNA has a  $k_{cat}/k_m$  of  $4 \times 10^5 \text{ min}^{-1} \text{ M}^{-1}$  with a  $K_m$  of about 50 nM. The activity of the Rz409<sub>(HPV11-E6/E7)</sub> against an HPV11 E6 target mRNA appears similar, although detailed analyses were not performed.

- Dz targeted to identified sites exhibited a spectrum of activities against <sup>32</sup>P-labeled HPV11 E6/E7 mRNA. For example, Dz27<sub>(HPV11-E6/E7)</sub> exhibited no *in vitro* catalytic activity, Dz338<sub>(HPV11-E6/E7)</sub> exhibited some *in vitro* activity, and Dz407<sub>(HPV11-E6/E7)</sub> exhibited good *in vitro* activity (Figure 4B). Interestingly, the 407 site was identified using both LS1 and LS2 protocols. In addition, out of the 12 Dz targeting the 12 sites identified with the LS2 protocol, Dz407<sub>(HPV11-E6/E7)</sub> was the only Dz showing good activity. The mutant Dz, Dz407m<sub>(HPV11-E6/E7)</sub>, did not exhibit any activity.

- Four single-stranded Dz library pools of varying lengths were obtained using an amplification reaction with ribose-containing primers (Figure 2): (1) Dz plus the TS and BS primer sequences (TS+Dz+BS; 67 nucleotides), (2) Dz plus the TS primer sequence (TS+Dz; 50 nucleotides), (3) Dz plus the BS primer sequence (Dz+BS; 50 nucleotides), and (4) Dz without the primer sequences (Dz; 33 nucleotides). Once obtained, they were tested for *in vitro* catalytic activity against labeled HPV16 E6/E7 mRNA. All four exhibited detectable cleavage activity at the targeted site. These results indicate that amplification reactions using ribose-containing primers can be used to obtain active Dz. These results also indicate that Dz of varying size can be used to cleave mRNA targets.

25

#### Example 4 - Cell culture analysis of ASO, Rz, and Dz

- The following cell culture experiments were performed using 293T human embryonic kidney cells (ATCC, Rockville, MD; # CRL-1573). The 293T cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) plus 10 percent heat-inactivated bovine calf serum.

30

In a preliminary stability study, 3'-protected (ASO<sub>p</sub>) or unprotected ASO were 5'-end labeled with  $\gamma^{32}\text{P}$ -ATP. The 5'- $^{32}\text{P}$ -labeled ASO<sub>p</sub> and unprotected ASO were then incubated for various periods in medium (DMEM plus 10 percent heat-inactivated bovine calf serum). 5'- $^{32}\text{P}$ -labeled ASO<sub>p</sub> and unprotected ASO also were transfected into 293T cells, and RNA was isolated on days 1, 3, and 5 following transfection.

In medium, no degradation of protected ASO<sub>p</sub> was observed at 48 hours. The unprotected ASO, however, exhibited about 70 to 80 percent degradation at 48 hours. After transfection into 293T cells, ASO<sub>p</sub> exhibited about three to five times more stability at day 5 than the degree of stability observed with unprotected ASO.

Cells stably expressing HPV11 E6/E7 mRNA were generated as follows. 293T cells were stably transfected using the Flp-In system (Invitrogen). The Flp-In 293T cells were grown in the presence of zeocin (400  $\mu\text{g/mL}$ ) to maintain the FRT site, which is used for targeted recombination at a unique site in the genome. An HPV11<sub>E6/E7</sub> sequence was cloned into a pcDNA5 vector, which was then co-transfected into the Flp-In 293 cells along with pOG44 (a vector that allows expression of the Flp recombinase) using Lipofectamine PLUS reagents. Two days after transfection, the cells were exposed to antibiotic selection with hygromycin B (30  $\mu\text{g/mL}$ ). This selected for the integrated pcDNA5/FRT construct. After 30 days, single colonies were picked, and tested for  $\beta$ -galactosidase activity and HPV11<sub>E6/E7</sub> expression. The stably transfected cells were designated 293T<sub>E6/E7</sub>. For comparative purposes, expression of HPV11<sub>E6/E7</sub> was about six times greater than that of HPV16<sub>E6/E7</sub> in CaSki cells.

The 293T<sub>E6/E7</sub> cells were subsequently transfected with various ASO, SNIP<sub>AA</sub> RZ constructs, or Dz targeted to HPV11 E6/E7. Transfection efficiencies were estimated to be about 70 to 90 percent. HPV11<sub>E6/E7</sub> target RNA levels were measured via real-time quantitative PCR (QPCR) using a Stratagene (La Jolla, CA) Mx4000 machine and TaqMan 5'-nuclease methodology, with either VIC or 6-carboxy-fluorescein (FAM) and Black Hole Quencher 1 (BHQ). For HPV11<sub>E6/E7</sub>, an initial 3'-primer with reverse complementary to nucleotides 827-803 was used. For QPCR, the 3'-primer was reverse complementary to nucleotides 226-203, and the 5'-primer matched nucleotides 154-175. The TaqMan probe was (FAM)-CTTTCCTTTGCAGCGTGTGCCTGT-(BHQ) (SEQ ID NO:72), which matched nucleotides 177-201.

mRNA for the ubiquitous transcription factor TATA-box binding protein (TBP, an integral component of TFIID) was used for normalization (Herasse *et al.*, *Mol. Cell Biol.*, 19:4047-55 (1999)). For TBP, the initial 3'-primer (5'-CTGGAAAACCCAACTT-CTGTACAA-3'; SEQ ID NO:73) was reverse complementary to nucleotides 742-718.

5 For QPCR, the same 3'-primer was used. The 5'-primer (5'-ACCACGGCACTGATT TTCAGT-3'; SEQ ID NO:74) matched nucleotides 625-646. The TaqMan probe ((VIC)-TGTGCACAGGAGCCAAGAGTGAAGA-(BHQ); SEQ ID NO:75) matched nucleotides 659-683. ROX (carboxy-X-rhodamine, succinimidyl ester) was used as dye for a volume control. In some experiments, 18S ribosomal RNA was also quantified.

10 RT reactions utilized Sensiscript reverse transcriptase (Qiagen) with 50 ng RNA. Standard curves were generally run with 25-100 ng total RNA. QPCR reactions used HotStar Taq Polymerase (Qiagen). 2  $\mu$ L of the RT reaction was used in a 20  $\mu$ L reaction. Amplifications were performed in triplicate, and HPV11<sub>E6/E7</sub> and TBP were matched from the same RT reactions for each. Initial denaturing was at 95°C for 15 minutes. Each  
15 cycle consisted of 95°C for 30 seconds, 56°C for 1 minute, and 72°C for 30 seconds. The data were analyzed using the REST program and the pair-wise fixed reallocation randomization test (Pfaffl *et al.*, *Nucleic Acids Res.*, 30:e36 (2002)) with TBP as reference. The results were similar without normalization.

One day following transfection, minor decreases in HPV11 E6/E7 mRNA levels  
20 were observed in cells transfected with SNIP dITRZ (409/409), SNIP dITRZ (409/244), SNIP dITRZ (409/162), SNIP ITRZ (409), SNIP ITRZ (244), and SNIP ITRZ (162). At 3 and 5 days following transfections, significant reductions in HPV11 E6/E7 mRNA were observed (Figure 5B). By day 5, the SNIP<sub>AA</sub> Rz constructs containing the dITRz were clearly more effective than those containing a single ITRz, producing reductions in  
25 HPV11 E6/E7 mRNA levels of about 80 to 90 percent. The greatest activity appeared to be attributable to Rz409 in a number of experiments. An empty SNIP cassette (i.e., no ITRz) had minor effects, and all SNIP<sub>AA</sub>Rz values differed significantly from control at day 3 and day 5. In various repeat experiments, similar results were obtained.

Cellular RNA was isolated from 293T<sub>E6/E7</sub> cells at 1, 2, and 3 days following  
30 transfection with Dz27<sub>(HPV11-E6/E7)</sub>, Dz338<sub>(HPV11-E6/E7)</sub>, Dz407<sub>(HPV11-E6/E7)</sub>, or Dz407m<sub>(HPV11-E6/E7)</sub>. After 1 day, cells transfected with each Dz constructs exhibited substantial

reductions in HPV11E6/E7 target mRNA levels (Figure 5B). Effects were similar at day 2, with all Dz constructs resulting in 50 to 60 percent reductions in target RNA levels. Effects were diminishing, but still evident, by day 3. The catalytically inactive mutant of Dz407<sub>(HPV11-E6/E7)</sub>, (Dz407m<sub>(HPV11-E6/E7)</sub>), resulted in effects that were equivalent (on day 5 3, even superior) to those of Dz407<sub>(HPV11-E6/E7)</sub>. In addition, Dz27<sub>(HPV11-E6/E7)</sub>, which is devoid of catalytic activity, resulted in major reductions in target RNA at all 3 time points. These results indicate that the activities observed under these conditions were attributable to antisense effects. Thus, the following *in vivo* studies included ASO directed to these sites.

10

#### Example 4 - Preparation of foreskin and orthografting

Foreskin fragments were prepared from circumcisions as described elsewhere (Slebos et al., PNAS, 91:5320-4 (1994)). The foreskin was free of detectable lesions and was cut split-thickness (0.5 mm), and then cut into 5 x 5 mm pieces. These pieces were 15 incubated with 0.2 mL of HPV-11 virus stock (Kreider *et al.*, *J. Virol.*, 61:590-3 (1987)) for 1 hour at 37°C, and then grafted immediately onto 5-10 week old female immunodeficient NIH-III mice (#NIHBNX-MMC, Harlan Laboratories, Indianapolis, IN). These mice carry three separate mutations (bg, nu, and xid), which result in deficiencies in T, B, and NK cells. The mice were anesthetized, and a section of epidermis/dermis 20 equivalent to the graft was removed over the rib cage. The graft was then placed in the area and covered with a 1x1 cm nonadherent dressing, which in turn was covered with porous tape wrapped around the mouse. The grafts were then allowed to heal for 10 to 14 days before the bandages were removed. The "take" rate was about 60 percent. Visible papillomas developed about 60 to 70 days following grafting. After infection and 25 grafting of foreskin, the various ASO or Dz were topically applied at the time papillomas became evident macroscopically (i.e., with diameters of 1-2 mm), ensuring that characteristic lesions would develop in each case. Spontaneous regression is not observed in this model. For treatment of grafts, the ASO or Dz were applied topically to developing papillomas in a volume sufficient to cover the surface (e.g., 25 µL, with DNA 30 at 1 µg/µL). Treatment was continued every other day for about 20 to 30 days, at which time the experiments were terminated, and the grafts were processed histology,

immunohistochemistry (for E6, E7, or L1 polypeptides), and via *in situ* hybridization (for HPV11 DNA).

Example 5 – Topical delivery of ASO and Dz

5           Topical delivery of expression constructs was quite poor, when tested with a variety of delivery vehicles, including cationic liposomes (Templeton *et al.*, *Nature Biotech.*, 15:647-52 (1997)). The delivery of smaller oligonucleotides (e.g., ASO and Dz) was assessed. In initial studies, fluorescein-labeled ASO or Dz were prepared in lipofectamine:ethanol (70:30, v:v) at concentrations of 1  $\mu\text{g}$  or 2  $\mu\text{g}/\mu\text{L}$ . ASO or Dz in  
10 lipofectamine:ethanol were applied topically to human foreskin or breast skin grafts, or to the back skin of 5-10 week old female NIH-III mice. Delivery was assessed by fluorescence microscopy at 6, 12, or 24 hours following topical application.

          Delivery of fluorescein-labeled ASO to full-thickness xenograft human epidermis and mouse epidermis was excellent using 1 or 2  $\mu\text{g}/\mu\text{L}$  DNA in a 70:30 mixture (v:v) of  
15 lipofectamine:ethanol, although it was not possible to document cellular uptake under these conditions. Delivery of fluorescein-labeled Dz was generally similar although somewhat more variable under the same conditions. In some experiments, PBS was used as delivery vehicle, and delivery appeared comparable, although delivery was poor after papillomas had developed.

20           In another experiment, fluorescein-labeled ASO (10  $\mu\text{L}$  of a 1  $\mu\text{g}/\mu\text{L}$  solution) were topically applied to mouse cervix. Two mice received fluorescein-labeled ASO with a gel foam, and two mice received the fluorescein-labeled ASO without the gel foam. The two mice receiving the ASO without the gel foam and one mouse receiving the ASO with the gel foam were sacrificed four hours after application. In each case,  
25 fluorescence was observed through the full-thickening of the cervical epithelium. The remaining mouse receiving the ASO with the gel foam was sacrificed twelve hours after application. In this case, no fluorescence was detected. In addition, no gel foam was detected, indicating that the gel foam may not have been properly applied or retained.



### Example 6 - *In situ* Hybridization and Immunohistochemistry

Cloned HPV11 (8 kbp, Hershey strain) was labeled with Bio-11-dUTP by random priming (Megaprime DNA Labeling System, Amersham, Piscataway, NJ) according to the manufacturer's instructions. Probes were diluted to 1  $\mu$ g/mL in a 50 % formamide hybridization cocktail (50% formamide in 2.4 M NaCl, 40 mM Tris-HCl, pH 7.4, 2 mM EDTA, 4 mg/mL BSA, 0.08% polyvinylpyrrolidone, 0.08% ficoll, 0.6 mg/mL yeast tRNA, and 80 mM dithiothreitol).

Paraffin sections were dewaxed, dehydrated with ethanol, and then digested with pepsin (4 mg/mL, in 0.1 N HCl). The tissues were then neutralized with 95 % ethanol, subsequently dehydrated in 100 % ethanol, and then allowed to air dry. The probe cocktail was applied to the tissue, and the tissue and probe combination was denatured at 95°C for 6 minutes. Hybridization was performed for 2 hours at 37°C in a moist chamber. The samples were then thoroughly washed in 2X SSC, and the hybridized probe was detected by incubation with an avidin-alkaline phosphatase conjugate, followed by colorimetric development in McGady reagent (nitroblue tetrazolium, 50 mg/mL, and 5-bromo-4-chloro-3-indoyl phosphate, p-toluidine salt, 50 mg/mL, in 50% dimethylformamide). Slides were counterstained with nuclear fast red and cover slipped in permanent mounting medium.

For immunohistochemical detection of E6 and E7 polypeptides, polyclonal antibodies were raised in chickens against three 17-aa polypeptides. The E7 polypeptide was HGRLVTLKDIVLDLQPC (SEQ ID NO:76). The two E6 polypeptides were MESKDASTSATSIDQLC (SEQ ID NO:77) and LELQGKINQYRHFNYAC (SEQ ID NO:78). These polypeptides were synthesized and HPLC purified (Genemed Biotechnologies, South San Francisco, CA). They were targeted to regions in HPV11 E6/E7, which were homologous to immunogenic regions previously identified in HPV16 (Stacey *et al.*, *Oncogene*, 9:635-45 (1994)). The polypeptides were coupled to keyhole limpet hemocyanin, and antibodies were induced by immunization of chickens on days 1, 21, 42, and 63 (Cocalico Biologicals, Reamstown, PA). After analysis of test bleeds, antiserum collected on day 73 was used, and antibodies were purified using thiophylc adsorption chromatography (T-gel absorbent, Pierce, Rockford, IL) according to the

manufacturer's specifications. Egg yolks were also obtained, and IgY immunoglobulins were purified from them.

For immunohistochemical staining, paraffin embedded tissues were used. Paraffin sections of cysts developed from HPV11-infected human foreskin, which had been grafted under the renal capsule of athymic mice, were used as a positive control. Immunoperoxidase staining was carried out using the avidin-biotin complex method (ABC kits, Vector Laboratories, Austin, TX). Sections were deparaffinized and hydrated through xylene and a graded ethanol series. Endogenous peroxidase was quenched using 3 % H<sub>2</sub>O<sub>2</sub> in water for 5 minutes. Blocking was performed with 10 % normal goat serum for 30 minutes, following by avidin-biotin blocking. The sections were incubated overnight at 4°C in a 1:100 dilution of T-gel purified antibodies, with 10 % normal goat serum in PBS, followed by 2 washes in PBS. Sections were subsequently incubated for 1 hour in biotinylated secondary goat anti-chicken IgG (8 µg/mL). Nova Red substrate (Vector Laboratories) was used as precipitating substrate for the localization of peroxidase activity, which was then visualized as red reaction product. In some experiments, a rabbit polyclonal antibody directed against HPV11 L1 (from Signet, Dedham, MA) was used with a goat anti-rabbit secondary antibody.

For immunoblot analyses, 15 % acrylamide gels were used (Laemmli, *Nature*, 227:680-5 (1970)). Nickel-purified histidine-tagged E6 fusion proteins and an HPV11 cyst extract were used as positive controls, and an HPV40 cyst extract was used as a negative control. Following PAGE, separated proteins were transferred to nitrocellulose membrane, and the membrane was blocked in Tween-20/Tris-buffered saline, and incubated with a 1:50 dilution of the antibody preparations in the same buffer. Controls included samples containing 15 µg/mL of the polypeptide used for immunization. Detection was performed using horseradish-peroxidase-linked rabbit anti-chicken IgG (Sigma, St. Louis, MO), with 4-chloro-1-naphthol and H<sub>2</sub>O<sub>2</sub> (each at 0.05 %), using methanol as chromogen.

#### Example 7 – Treating HPV infections

For *in vivo* studies, a model was used wherein human foreskin, obtained locally from a consortium of hospitals/physicians, was infected with HPV11 and grafted onto

NIH-III triple immunodeficient mice (Kreider *et al.*, *J. Virol.*, 61:590-3 (1987)). In this model, papillomas develop and become visible grossly after a characteristic incubation period of about 60 days. After infection and grafting of foreskin, topical application of various ASO or Dz was begun at the time papillomas became evident macroscopically.

5 Treatment was continued every other day for about 20-30 days, at which time the experiments were terminated, and the grafts were processed for histology, immunohistochemistry (for E6, E7, or L1 protein), and via *in situ* hybridization (for HPV11 DNA). In total, 21 foreskin grafts were treated with Dz407<sub>(HPV11-E6/E7)</sub> (n=10) and ASO407<sub>(HPV11-E6/E7)</sub> (n=11; 6 unprotected and 5 protected), excluding various controls.

10 The largest and most consistent therapeutic responses were observed with ASO407<sub>(HPV11-E6/E7)</sub>, either as a protected (3'-inverted T) or an unprotected ASO (Table 2). In 4 of 11 grafts treated with ASO407<sub>(HPV11-E6/E7)</sub>, a complete therapeutic success was achieved in that no residual papillomatous lesions remained and no residual HPV11 could be detected in the grafts by *in situ* hybridization. In one graft treated with ASO407<sub>(HPV11-</sub>  
 15 <sub>E6/E7)</sub>, no residual HPV11 could be detected by *in situ* hybridization, although gross evidence of the papilloma remained at the end of treatment. This papilloma had a slight reduction in size (-4 % and -12% at 14 and 28 days, respectively) in contrast to grafts with persistent virus, all of which consistently demonstrated increases in papilloma size. Eradication of viral DNA was thus achieved in 5 of 11 grafts treated with ASO407<sub>(HPV11-</sub>  
 20 <sub>E6/E7)</sub> compared to 0 of 6 control grafts. Using Fisher's exact test, there is no significant difference in the proportion of viral negativity between the ASO-treated samples and controls (p=0.10), which presumably reflects the small sample size. In addition to the complete responses, partial responses were observed histologically in the remaining 6 of 11 ASO407<sub>(HPV11-E6/E7)</sub>-treated grafts, consisting of localized reductions in *in situ*  
 25 hybridization signal. These, however, were not accompanied by reductions in the size of the papillomas.

Table 2. Complete response rates by *in situ* hybridization (ISH) of treated foreskin grafts.

Reagent Applied	# grafts ISH Positive	# grafts ISH Negative	Total # grafts	Complete ISH Response Rate (%)
ASOp407 <sub>(HPV11-E6/E7)</sub>	6	5	11	45
Dz407 <sub>(HPV11-E6/E7)</sub>	7	3	10	30

Treatment effects in partial responders appeared to be inversely related to the extent of hyper- and parakeratosis within local regions within the papillomas. In this regard, marked residual viral staining was evident in areas showing the thickest cornification. This result indicates that treatment efficacy can reflect the ability of the ASO to penetrate the keratin layer. There was no clear enhancement of activity with the protected ASOp407<sub>(HPV11-E6/E7)</sub>, in spite of the significant increase in stability observed *in vitro*. Immunohistochemistry for HPV11 E7 polypeptides also confirmed concurrent loss of E6/E7 polypeptides under these conditions.

In the Dz407<sub>(HPV11-E6/E7)</sub>-treated grafts, 3 of 10 grafts had no evidence of HPV11 DNA using *in situ* hybridization (Table 2). In each of these cases, however, residual papillomas were observed grossly following treatment, and reductions in gross papilloma size were not noted. Apoptotic cells were often evident, with accumulation of nuclear debris. This response was particularly prominent in samples treated with Dz407<sub>(HPV11-E6/E7)</sub>, and again may reflect partial penetration and/or uptake of Dz, even in areas with relatively little keratinization. This cellular response was often accompanied by a notable loss of koilocytes. In 7 of 10 grafts demonstrating positive *in situ* hybridization, microscopic effects were variably observed, but were restricted to the upper layers of the epidermis.

Grafts also were treated with Dz27<sub>(HPV11-E6/E7)</sub>, Dz338<sub>(HPV11-E6/E7)</sub>, and Dz407m<sub>(HPV11-E6/E7)</sub>. Treatment with Dz27<sub>(HPV11-E6/E7)</sub> produced slight therapeutic effects, even though Dz27 exhibited no catalytic activity when tested *in vitro*. This therapeutic effect can be attributed to an antisense effect. Treatment with Dz338<sub>(HPV11-E6/E7)</sub> produced variable results. In some grafts, a complete blockage of papilloma development was observed. Grafts treated with Dz407m<sub>(HPV11-E6/E7)</sub> also showed partial therapeutic effects.

These studies involved developing and applying library selection protocols to identify accessible sites in HPV target mRNA, namely the E6/E7 bicistronic mRNA. Both the LS1 selection protocol and the LS2 protocol employed iterative binding steps to enrich the libraries for accessible binding regions. LS1 employs a library of random oligonucleotides and identifies sites accessible for binding, whereas LS2 employs a library of random Rz and directly identifies Rz cleavage sites.

The 407/409 cut site region was identified in both LS1 and LS2 protocols. This region is homologous to the nucleotide position 427 region in HPV 16<sub>E6/E7</sub> mRNA, one of the HPV types important in cervical cancer. In HPV16<sub>E6/E7</sub> mRNA, sites/regions 434 or 437-447 were previously described by others (Alvarez-Salas *et al.*, *Antisense Nucleic Acid Drug Dev.*, 9:441-50 (1999) and Venturini *et al.*, *Nucleic Acids Res.*, 27:1585-92 (1999)). Since different numbering schemes were used, the 434 site identified by others corresponds to site 358 described herein (as a hammerhead ribozyme, Rz358 would cleave after nucleotide 436 in the alternate numbering scheme). Rz358 was in fact identified in an HPV16<sub>E6/E7</sub> LS1 protocol. In our hands, however, Rz358 activity was slightly less than that of Rz427 *in vitro*, and Rz427 exhibited superior effects in cell culture. The 427 site in HPV16<sub>E6/E7</sub> would correspond to nucleotide 505 in the alternate numbering scheme.

As described herein, Dz produced substantial reductions in HPV11<sub>E6/E7</sub> target RNA in cell culture, in spite of the relatively high target expression levels (and <100 % transfection efficiencies). However, since catalytically inactive Dz produced equivalent results, ASO targeted to the selected sites were also included in subsequent testing *in vivo*.

In addition, ASO and Dz constructs were also tested with 3'-inverted T's as protective groups. The 3'-inverted T constructs showed significantly increased stability in medium and within cells. However, the 3'-inverted T modification did not increase the efficiency of Dz in cell culture under the conditions utilized, and more importantly it had no demonstrable effect on the efficacy of ASO *in vivo*. Enhanced activity with 3'-inverted T moieties might become apparent at lower doses or with extended intervals between applications.

Papillomas in humans are known to sporadically undergo spontaneous resolution. It is thought that this phenomenon is immunologically-mediated. During the 8-week observation period in studies of genital papillomas treated with imiquimod, 0/57 control lesions underwent spontaneous regression (Beutner *et al.*, *J. Am. Acad. Dermatol.*, 38:230-239 (1998)). During the experiments described herein using the human foreskin graft model, no papillomas exhibited evidence of spontaneous regression during the observation period. This is further supported by the persistence and growth of papillomas

in control grafts. Furthermore, since spontaneous regression of papillomas is immunologically-mediated, this phenomenon would not be expected to occur in immunodeficient mice.

Grafts treated with ASO407<sub>(HPV11-E6/E7)</sub> exhibited the greatest therapeutic efficacy, completely eliminating viral DNA expression in 5 of 11 samples, causing complete regression of papillomas in 4 of these 5. The elimination of viral expression was statistically significant ( $p < 0.02$ ) based on comparison with concurrent and historic foreskin graft controls. This complete response ("cure") rate of 4 of 11 (36 %) is comparable to the rate achieved with topical imiquimod and other therapies (Beutner *et al.*, *J. Am. Acad. Dermatol.*, 38:230-239 (1998)). The remaining 7 of 11 grafts (64 %) treated with ASO407<sub>(HPV11-E6/E7)</sub> exhibited partial responses.

Dz407<sub>(HPV11-E6/E7)</sub>, and other less active Dz targeted to other library selected sites, also exhibited a therapeutic efficacy, at least microscopically, in terms of viral eradication and cellular effects. In 3 of 10 grafts, viral DNA expression was abolished, yet gross evidence of the papilloma remained at the end of therapy. It is possible that there may be a lag time between viral eradication and regression of the papilloma. Since the animals were sacrificed at the time the grafts were harvested, this hypothesis was not addressed in these experiments.

The therapeutic effects of Dz *in vivo* appeared to be restricted to the upper levels of the epidermis, where apoptosis and nuclear debris were observed, as well as absence of koilocytosis. This effect was greatest with Dz407<sub>(HPV11-E6/E7)</sub>, and appeared to be specific, since it was not observed after treatment with irrelevant Dz, DzAR2000. Restriction to the upper levels of epidermis might reflect poorer penetration of Dz as compared to the penetration of ASO (32 nucleotides for protected Dz, 31 nucleotides for unprotected Dz, 18 nucleotides for protected ASO, and 17 nucleotides for unprotected ASO), although this was not clearly supported in topical delivery studies with fluorescein-labeled oligonucleotides, or it might reflect lower cellular uptake of Dz.

The partial responses to eradication of HPV viral DNA observed with both ASO and Dz clearly correlated with the local thickness of the keratin layer. *In situ* hybridization for HPV11 DNA showed strong staining in areas exhibiting extensive keratinization in residual lesions, and often showed little or no residual staining in areas

with little keratinization. The extent of keratinization thus appears to affect penetration, which could contribute to the reduced efficacy in areas of extensive hyperkeratosis.

Improved results can be obtained in early papillomas, which are less extensively keratinized. This may not represent an obstacle when treating mucosal surfaces.

- 5 Alternatively, for nonmucosal skin, these reagents can be combined with a pretreatment aimed at reducing the thickness of the stratum corneum such as the use of topical salicylic acid, paring, or cryotherapy.

The results provided herein demonstrate the successful treatment of HPV-induced lesions. These results also demonstrate that nucleic acid agents such as ASO, siRNA  
10 molecules, Rz, and Dz can be used to reduce the number of cells infected with HPV *in vivo*. In fact, successful targeting of HPV11<sub>E6/E7</sub> mRNA can block papilloma development in human foreskin, often with elimination of detectable HPV. Efficacy appears to be inversely related to the extent of keratinization, so that clinical applications on nonmucosal skin can be coupled with treatments to reduce and/or eliminate surface  
15 cornification prior to (or during) topical delivery.

#### Example 8 - Library selection of HPV16-E6/E7 target sites

A double-stranded DNA library was used to generate an Rz-library with multiple copies of about  $10^{10}$  different RNA sequences. Each transcript was 79 nucleotides in  
20 length, with a central Rz core flanked on each side by random sequences of 9Ns and by defined 5'- and 3'-end sequences. See, International Patent Application No. PCT/US01/46178. The main purposes of the fixed 5' and 3' sequences were 2-fold: (1) they allow a PCR-based iterative protocol for regeneration of bound species; (2) they substantively decrease (by 20X) the catalytic activity of the active Rz species, which  
25 results in identification of Rz that are highly active in subsequent testing; and (3) they facilitate cloning into the SNIP cassette.

DNA templates of targeted RNA were generated by RT-PCR with a T7 promoter in the 5'-primers. To circumvent the problem of microheterogeneity of transcripts at their 3'-ends, a 3'-primer encoding a self-cleaving Rz was used so that transcripts with precise  
30 3'-GUC ends were produced during *in vitro* runoff transcription.

Incubating the active Rz library with target RNA did not yield cleavage products, due to the huge diversity engineered into the library. To circumvent this, the Rz library was first subjected to selection under magnesium-free conditions, to allow isolation of RNA molecules that annealed to the corresponding target-RNA. The isolated annealed  
5 Rz-library RNA pool was subsequently amplified by RT-PCR, and then subjected to a second round of selection at a lower target-RNA ratio, to increase the selection stringency, and to decrease background. The annealing temperature of 85° C was used to maximize binding of library Rz to all regions of the target RNA during these binding steps. When annealing was performed at 37°C, the procedure did not work. This might  
10 be attributable to the lack of magnesium in the incubations.

The re-amplified second round selected Rz (sRz) library RNA pool was used to cleave 5'- or 3'-end labeled target-RNA. The cleaved products were analyzed on sequencing gels, in comparison with G-, A-, and/or base-hydrolysis products, and the cleavage sites were precisely identified. Eleven cleavage sites were located on the  
15 HPV16 E6/E7 target RNA. The intensity of the cleaved products generally (but not always) reflected the catalytic activity of that sRz. Occasional anomalous bands were observed, presumably reflecting changes in site accessibility due to interplay between distinct Rz. Without the iterative binding steps, no cleavage of target RNA could be identified.

20 Within HPV16 E6/E7 RNA, sites/regions 434 (Alvarez-Salas *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 95:1189-1194 (1998)) or 437-447 (Venturini *et al.*, *Nucleic Acids Res.*, 27:1585-1592 (1999)) have been previously described. Given the different numbering schemes, the 434 site identified corresponds to site 358 herein (as an Rz, this would cleave after nucleotide 436 in the alternate numbering scheme). Rz358 was in fact  
25 identified in an HPV16 E6/E7 LS1 protocol. In addition, Rz358 activity was slightly less than that of Rz427 *in vitro*, but Rz427 showed superior effects in cell culture.

#### Example 9 - *In vitro* cleavage kinetics of sRz targeting HPV16 E6/E7 mRNA

The catalytic activities of sRz were determined using single turnover conditions.  
30 Briefly, 10 nM of 5'-<sup>32</sup>P-labeled target RNA was incubated with 40 nM Rz in buffer containing varying concentrations of MgCl<sub>2</sub> (1, 5, or 25 mM) at 37° C for 30 minutes. 25



mM MgCl<sub>2</sub> was used because it has been reported to yield cleavage rates similar to those observed in the presence of cytosol (Nedbal and Sczakiel, *Antisense Nucleic Acid Drug Dev.*, 7:585-9 (1997)). Cleavage products were then analyzed by denaturing PAGE (Figure 6). Eight of 11 of the sRz targeted to HPV16 E6/E7 mRNA exhibited even greater activity than Rz427, the most active Rz identified using the LS1 oligonucleotide library procedure (Pan *et al.*, *RNA*, 7:610-621 (2001)). Two others exhibited activity roughly comparable activity exhibited by Rz427. Nearly all of the sRz were "highly efficient" according to previous categorizations (Pan *et al.*, *RNA*, 7:610-621 (2001)). The "highly efficient" category reflects cleavage of about 40 to 50 % of target RNA after 30 minutes at 37°C with 40 nM Rz concentration. With the higher concentrations of magnesium, sRz catalytic activities were increased, but the differential between them was decreased somewhat. For example, compared with Rz427, the best selected sRz (sRz59) was 2.4 times more active in 25 mM MgCl<sub>2</sub> and 4.5 times more active in 5 mM MgCl<sub>2</sub>. In addition, only sRz59 and sRz68 exhibited demonstrable activity with 1 mM MgCl<sub>2</sub>. Even when the concentration of sRz59 was reduced to 1 nM in reactions containing 10 nM of target, cleavage products were still visible after PAGE and autoradiography (Figure 6B).

Analysis of the efficient cleavage sites for HPV16 E6/E7 target RNA revealed a tendency for a GUA-triplet, similar to that for other Rz, with GUU and GUC exhibiting slightly lower prevalence (Table 3). A distinguishing feature was two "D" nucleotides ("A", "G", or "U", but not "C") surrounding the triplet, yielding a consensus of 5'-DRUHD-3'. More extended analyses with other target RNAs yield a similar consensus of 5'-DNUHD-3', with the "N" being "G" nearly two thirds of the time.

Table 3. Analysis of nucleotide availability surrounding cleavage sites

Position	16.10	16.9	16.8	16.7	16.6	16.5	16.4	16.3	16.2	16.1	17	1.1	1.2	1.3	1.4	1.5	1.6
Adenosine	1	4	1	5	2	2	3	3	1		4	2	4	0	4	3	4
Cytidine	2	1	2	1	1	1	2	0	0		2	0	1	1	1	2	3
Guanosine	2	0	1	2	4	2	2	3	7		0	3	2	4	3	1	1
Uridine	3	3	4	0	1	3	1	2	0	8	2	3	1	3	0	2	0

After identification of efficient cleavage sites in the various target RNAs, the nucleotides in positions surrounding the NUH triplet were compiled. "Position" indicates the nucleotide positions 3-10 in the complementary flanking sequence upstream of the NUH

triplet in Helix III (16.x), and nucleotide positions 1-6 in the complementary flanking sequence downstream of the NUH triplet (1.x). Nucleotide positions are numbered as follows with the cleavage site being located between the underlined nucleotides (numbered 17 and 1.1):

5 (16.10 ----- 16.1)17 (1.1 ----- 1.9)  
5'-N N N N N N N N N U H N N N N N N N N N N-3' Target

10 The HPV16 E6/E7 target RNA was modeled using the mFold program (Figure 7; the locations of the sRz sites are denoted with arrows). In general, sRz sites are found at "transitions", where one flanking region is in a region predicted to be double-stranded, and the other flanking region is in a region predicted to be single-stranded. Surprisingly, sRz sites were rarely (if ever) found where the flanking sequences are predicted to both  
15 be in single-stranded regions (based on identification of about 50 sites in various targets). In fact, three Rz targeted to such sites in HPV16 E6/E7 RNA using mFold (mRz; Figure 7) were constructed and found to be inactive (mRz406 and mRz565) or found to exhibit very low activity (mRz327).

For kinetic analyses, 40 nM Rz and 10-1000 nM of target RNA were incubated  
20 for various periods (ranging from 40 seconds to 60 minutes) to obtain kinetic data for both single and multiple turnover conditions. Results for the HPV16 E6/E7 targeted sRz generally revealed  $K_m$ 's of about 20 to 80 nM. sRz59 exhibited a  $k_{cat}/K_m$  of  $1.52 \times 10^7$  ( $M^{-1} \text{ min}^{-1}$ ), a value about 5 times higher than Rz427's  $k_{cat}/K_m$  value of  $2.84 \times 10^6$  ( $M^{-1} \text{ min}^{-1}$ ). This presumably reflects a faster chemical step of sRz59's catalytic activity, since  
25  $K_m$  values appear to be similar.

#### Example 10 – Cell culture analysis of sRz targeting HPV16 E6/E7 mRNA

The sRz targeted to HPV16 E6/E7 RNA were placed within the SNIP<sub>AA</sub> cassette as described herein. The HPV16-E6/E7 target construct and the SNIP<sub>AA</sub>sRz constructs  
30 were co-transfected into 293T cells, and cultures were analyzed for the effects of sRz on HPV16-E6/E7 RNA expression. At 3 and 5 days after transfection with the SNIP<sub>AA</sub> constructs containing sRz (sRz59, sRz68, sRz187, sRz251, and sRz275) or with SNIP<sub>AA</sub>-Rz427 (the most active Rz identified using the LS1 procedure), a substantial reduction of HPV16 E6/E7 RNA was observed (Figure 8A). The largest reduction was produced by

SNIP<sub>AA</sub>-sRz59 at day 3. By day 5, the E6/E7 RNA transcripts were reduced by 75 % in all of the sRz transfections, compared with co-transfections with the empty SNIP<sub>AA</sub> cassette.

In repeat co-transfection experiments, QPCR was used to measure HPV16 E6/E7 mRNA levels (Figure 8B). All the SNIP<sub>AA</sub>sRz constructs were very effective at reducing HPV16 E6/E7 target RNA levels, achieving reductions of about 60 to 80 % on day 5. Essentially all reductions in HPV16 E6/E7 RNA levels were statistically significant at  $p < 0.001$ . Statistically significant reductions were also evident on day 1 and day 7, with average decreases of 34 % and 47 %, respectively. This reductions shown in Figure 8B were slightly greater than the reductions observed with similar SNIP<sub>AA</sub>-Rz cassettes designed to express Rz targeting selected sites in HPV11 E6/E7 target RNA, but were smaller than the 90 % reductions observed with SNIP<sub>AA</sub> constructs containing double ITRz. In contrast, parallel experiments with SNIP<sub>AA</sub> constructs containing catalytically inactive versions of Rz59 and Rz427 (mRz59 and mRz427) did not produce significant reductions in HPV16 E6/E7 RNA transcripts (Figure 8C).

Growth experiments were conducted with SiHa cells (a human cell line derived from a cervical squamous cell carcinoma). SiHa cells contain an integrated copy of HPV16, and their growth is dependent, at least in part, upon continued expression of HPV16 E6/E7 transcripts (Rorke, *J. Natl. Can. Inst.*, 89:1243-1246 (1997); Madrigal *et al.*, *Gyn. Onc.*, 64:18-25 (1997); and Tan and Ting, *Cancer Res.*, 55:4599-4605 (1995)). While this protocol necessitates selection of growth-inhibited cells, it nevertheless provides a model system with sustained E6/E7 expression.

SiHa cells were transfected with the pCMV/BSD plasmids containing the various sRz in the SNIP<sub>AA</sub> cassette, and the transfected populations were selected for antibiotic resistance (with blastocidin S at 10  $\mu\text{g/mL}$ ). Selection was complete after 6 days. After 8 days, cells were counted in triplicate samples. Populations transfected with the 59, 68, or 251 SNIP<sub>AA</sub>sRz constructs exhibited 40 to 45 % reductions in cell growth ( $p < 0.05$ ), compared with cells transfected with the empty SNIP<sub>AA</sub> cassette or with a reporter expression construct. These reductions correspond to "maximal reductions" observed in other studies (Madrigal *et al.*, *Gyn. Onc.*, 64:18-25 (1997) and Tan and Ting, *Cancer Res.*, 55:4599-4605 (1995)). It is possible that an 80 % reduction in E6/E7 RNA

expression levels still provides sufficient E6/E7 polypeptide to sustain growth at a 50 % rate. It, however, also is possible that growth of SiHa cells is now only partially dependent upon E6/E7 expression. Cells transfected with the previously identified SNIP<sub>AA</sub>-Rz427, and those transfected with the 187 or 275 SNIP<sub>AA</sub>sRz constructs, exhibited more modest reductions (up to 15 %) in growth rates. In repeat experiments, analysis of RNA by QPCR revealed equivalent reductions in HPV16 E6/E7 target RNA on day 5. In other experiments with SNIP<sub>AA</sub> constructs containing double ITRz, reductions of about 60 to 75 % at days 7 and 12 were observed.

In summary, the LS2 library-screening procedure provides a relatively straightforward and rapid method for determining efficient cleavage sites in long, structured target RNAs. The iterative procedure was streamlined to 2 rounds of selection, and the entire procedure can be finished in a few days. A significant number of effective cut sites are generally identified. Most sRz identified are “highly efficient” *in vitro*. This in part reflects the fact that their activity within the library pool is considerably decreased by the presence of fixed 5'- and 3'- sequences, ensuring major increases in activity when they are constructed and tested without the extraneous flanking sequences. More importantly, the selected Rz targeted to HPV16 E6/E7 RNA were highly effective in cell culture models, and they can be used in *in vivo* models. These results demonstrate that the LS2 method is relatively simple, iterative Rz-library screening method that can be used to design hammerhead ribozymes.

#### Example 11 – Analysis of Rz and Dz specificities targeted to Hepatitis B Virus (HBV) RNA

Various mutant Rz and Dz (mutants of Rz885<sub>(HBV)</sub> and Dz879<sub>(HBV)</sub>; Pan *et al.*, RNA, 7:610-621 (2001) and International Patent Application No. PCT/US01/46178) were tested to assess the affects of mutations on the ability of the Rz or Dz to interact with and cleave a target RNA. Mutant forms of Rz885<sub>(HBV)</sub> and Dz879<sub>(HBV)</sub> were synthesized and incubated with HBV RNA transcripts (Pan *et al.*, RNA, 7:610-621 (2001)). The mutations were introduced into the N or Q regions of the Rz or Dz (Figure 9A). The nucleotide numbering is set forth in Figure 9A. To generate the mutants, a “G” was replaced with an “A”; an “A” was replaced with a “G”; a “C” was replaced with a “T” or

“U”; and a “T” or “U” was replaced with a “C.” Many mutations reduced the activity of the Rz and Dz (Table 4 and Figure 9B). Some mutations increased the catalytic activity of the Rz and Dz (Table 4 and Figure 9B).

5 Table 4. Relative activity for mutant Rz and Dz.

Lane in Figure 9B	Rz/Dz mutation	% Relative Activity of Rz	% Relative Activity of Dz
1	No Rz or Dz	0	0
2	Wild type	100	100
3	N <sub>1</sub>	0	0
4	Q <sub>1</sub>	14	57
5	N <sub>1</sub> +Q <sub>1</sub>	0	1
6	N <sub>2</sub>	41	0
7	Q <sub>2</sub>	2	19
8	N <sub>2</sub> +Q <sub>2</sub>	7	0
9	N <sub>1</sub> +2	4	0
10	Q <sub>1</sub> +2	0	5
11	N <sub>3</sub>	110	11
12	Q <sub>3</sub>	38	17
13	N <sub>3</sub> +Q <sub>3</sub>	48	0
14	N <sub>2</sub> +3	0	0
15	Q <sub>2</sub> +3	1	0
16	N <sub>4</sub>	102	17
17	Q <sub>4</sub>	59	17
18	N <sub>4</sub> +Q <sub>4</sub>	64	0
19	N <sub>3</sub> +4	57	0
20	Q <sub>3</sub> +4	8	5
21	N <sub>5</sub>	88	110
22	Q <sub>5</sub>	77	20
23	N <sub>5</sub> +Q <sub>5</sub>	72	23
24	N <sub>4</sub> +5+Q <sub>4</sub> +5	5	0

#### Example 12 – Cell culture analysis of siRNA targeting HPV16 E6/E7 mRNA

10 Constructs containing cassettes that express RNA molecules designed to induce RNA interference against HPV16 mRNA were made and tested in Flip293 cells as described elsewhere (U.S. Provisional Patent Application No. 60/449,066). In particular, the following cassettes were tested one day after transfection for the ability to reduce HPV16 mRNA levels within cells when driven by a CMV promoter sequence:

p1CLIP<sub>S</sub>/HPV16<sub>C+50-68+GUU</sub>; p1CLIP<sub>AS</sub>/HPV16<sub>C+68-50+GUU</sub>; pSIR/HPV16<sub>SiC+50-68+GUU</sub>;

p1CLIP<sub>HP</sub>/HPV16<sub>C+50-68+GUU</sub>; pSIR<sub>HP</sub>/HPV16<sub>C+50-68+GUU</sub>; p2CLIP<sub>HP</sub>/HPV16<sub>C+50-68+GUU</sub>; p2CHOP<sub>HP</sub>/HPV16<sub>C+50-68+GUU</sub>; pSNIP<sub>HP</sub>/HPV16<sub>C+50-68+GUU</sub>; p2CLIP<sub>HR</sub>/HPV16<sub>C+50-68+GUU</sub>; p2CHOP<sub>HR</sub>/HPV16<sub>C+50-68+GUU</sub>; pSNIP<sub>HR</sub>/HPV16<sub>C+50-68+GUU</sub>; and pSNIP (empty cassette). In addition, the following cassettes were tested two days after transfection for the ability to reduce HPV16 mRNA levels within cells when driven by a U6 promoter sequence: p1CLIP<sub>S</sub>/HPV16<sub>C+50-68+GUU</sub>; p1CLIP<sub>AS</sub>/HPV16<sub>C+68-50+GUU</sub>; pSIR/HPV16<sub>siC+50-68+GUU</sub>; p1CLIP<sub>HP</sub>/HPV16<sub>C+50-68+GUU</sub>; pSIR<sub>HP</sub>/HPV16<sub>C+50-68+GUU</sub>; p2CLIP<sub>HR</sub>/HPV16<sub>C+50-68+GUU</sub>; p2CHOP<sub>HR</sub>/HPV16<sub>C+50-68+GUU</sub>; pSNIP<sub>HR</sub>/HPV16<sub>C+50-68+GUU</sub>; pSNIP cassette containing sRz59; and pSNIP (empty cassette).

Significant reductions in target mRNA were detected for cells containing the pSIR/HPV16<sub>siC+50-68+GUU</sub> cassette or the pSIR<sub>HP</sub>/HPV16<sub>C+50-68+GUU</sub> cassette when the cassette was expressed from a CMV promoter sequence (Figure 10A). A significant reduction in target mRNA also was detected for cells containing the pSIR/HPV16<sub>siC+50-68+GUU</sub> cassette, the pSIR<sub>HP</sub>/HPV16<sub>C+50-68+GUU</sub> cassette, or the pSNIP<sub>HR</sub>/HPV16<sub>C+50-68+GUU</sub> cassette when the cassettes were expressed from a U6 promoter sequence (Figure 10B).

These results demonstrate that cassettes can be designed to express RNA sequences that induce RNA interference and reduce HPV16 mRNA levels within cells.

#### Example 13 – Kinetic analysis of Rz and Dz

The kinetics of Rz59<sub>(HPV16 E6/E7)</sub>, Dz57<sub>(HPV16 E6/E7)</sub>, and Dz589<sub>(HPV16 E6/E7)</sub> were determined using HPV16 E6/E7 RNA as the target (Table 5). Rz59<sub>(HPV16 E6/E7)</sub> and Dz57<sub>(HPV16 E6/E7)</sub> target cleavage sites that were identified using the LS2 protocol, while Dz589<sub>(HPV16 E6/E7)</sub> targets a cleavage site that was identified using the LS3 protocol. These results demonstrate that Rz and Dz can be designed to have high levels of catalytic activity. In addition, the measured  $k_m$  values indicate that these Rz and Dz can function at a high level within cells.

Table 5. Kinetic analysis of Rz and Dz cleavage of target RNA.

Rz/Dz	$k_m$ (nM)	$k_{cat}$ (min <sup>-1</sup> )	$k_{cat}/k_m$ (M <sup>-1</sup> · Min <sup>-1</sup> )
Rz59	21.05	0.32	1.52 X 10 <sup>7</sup>
Dz57	97.64	0.35	3.58 X 10 <sup>6</sup>
Dz589	21.46	2.69	1.25 X 10 <sup>8</sup>

### OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.

- 5 Other aspects, advantages, and modifications are within the scope of the following claims.

**WHAT IS CLAIMED IS:**

1. A method of treating HPV infection comprising administering an effective amount of a nucleic acid molecule to a patient in need thereof wherein said nucleic acid molecule  
5 inhibits expression associated with HPV replication.
2. The method of claim 1, wherein said HPV is HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV52, or HPV58.
- 10 3. The method of claim 1, wherein said nucleic acid molecule is complementary to a nucleic acid sequence of said HPV.
4. The method of claim 1, wherein said nucleic acid molecule is administered topically.  
15
5. The method of claim 4, wherein said nucleic acid molecule is administered topically to a portion of the genital organ of said patient.
6. The method of claim 4, wherein said nucleic acid molecule is administered  
20 topically to cervical tissue of said patient.
7. The method of claim 1, wherein said nucleic acid molecule is an antisense oligonucleotide or an siRNA molecule.
- 25 8. The method of claim 1, wherein said nucleic acid molecule is a ribozyme or DNzyme.
9. The method of claim 1, wherein said nucleic acid molecule is administered together with a keratolytic agent, wherein said keratolytic agent is present in an amount  
30 effective to enhance the penetration of said nucleic acid molecule.



10. The method of claim 9, wherein said keratolytic agent is salicylic acid or one or more alpha hydroxy acids.
11. The method of claim 1, wherein said patient suffers from cervical intraepithelial dysplasia (CIN).  
5
12. The method of claim 11, wherein said CIN is CIN I or mild dysplasia.
13. The method of claim 11, wherein said CIN is CIN II or moderate to marked dysplasia.  
10
14. The method of claim 11, wherein said CIN is CIN III or severe dysplasia to carcinoma-in-situ.
- 15 15. The method of claim 14, wherein said carcinoma-in-situ is localized to the intraepithelial tissue or the superficial layer of the cervix.
16. The method of claim 1, wherein said nucleic acid molecule is identified using a library selection technique.  
20
17. The method of claim 16, wherein said library selection technique is an antisense oligonucleotide library selection technique.
18. The method of claim 16, wherein said library selection technique is a ribozyme library selection technique.  
25
19. The method of claim 16, wherein said library selection technique is a DNase library selection technique.
- 30 20. A method for treating a mammal having cells infected with HPV, said method comprising administering a nucleic acid molecule to said mammal under conditions

wherein the number of said cells infected with said HPV is reduced, wherein said nucleic acid molecule comprises a sequence complementary to a nucleotide sequence of said HPV.

5     21.     The method of claim 20, wherein said mammal is a non-human, immunodeficient mammal, and wherein said cells are human cells.

22.     The method of claim 20, wherein said mammal is a nude or SCID mouse.

10    23.     The method of claim 20, wherein said mammal is a human.

24.     The method of claim 20, wherein said cell is a skin cell or epithelial cell.

25.     The method of claim 20, wherein said HPV is HPV11, HPV16, HPV18, HPV31,  
15    HPV33, HPV35, HPV39, HPV45, HPV52, or HPV58.

26.     The method of claim 20, wherein said nucleic acid molecule is administered topically to said mammal.

20    27.     The method of claim 26, wherein said nucleic acid molecule is administered topically to a portion of the genital organ of said mammal.

28.     The method of claim 26, wherein said nucleic acid molecule is administered topically to cervical tissue of said mammal.

25    29.     The method of claim 20, wherein said nucleic acid molecule is an antisense oligonucleotide or an siRNA molecule.

30    30.     The method of claim 20, wherein said nucleic acid molecule is a ribozyme or DNzyme.

31. The method of claim 20, wherein the number of said cells infected with said HPV is reduced by at least 25 percent.
32. The method of claim 20, wherein the number of said cells infected with said HPV is reduced by at least 50 percent.
33. The method of claim 20, wherein the number of said cells infected with said HPV is reduced by at least 75 percent.
34. The method of claim 20, wherein said nucleotide sequence comprises a target site identified using a library selection technique.
35. The method of claim 34, wherein said library selection technique is an antisense oligonucleotide library selection technique.
36. The method of claim 34, wherein said library selection technique is a ribozyme library selection technique.
37. The method of claim 34, wherein said library selection technique is a DNAzyme library selection technique.
38. The method of claim 20, wherein said cells infected with said HPV contain non-integrated HPV nucleic acid.
39. The method of claim 20, wherein said cells infected with said HPV contain replicating HPV.
40. An isolated catalytic nucleic acid comprising a catalytic core sequence, a 5' recognition sequence, and a 3' recognition sequence, wherein said isolated catalytic nucleic acid cleaves a target mRNA sequence selected from the group consisting of the sequences set forth in SEQ ID NOs:4-48.

41. The isolated catalytic nucleic acid of claim 40, wherein said isolated catalytic nucleic acid is a ribozyme.

5 42. The isolated catalytic nucleic acid of claim 41, wherein said catalytic core sequence comprises the sequence set forth in SEQ ID NO:51.

43. The isolated catalytic nucleic acid of claim 40, wherein said isolated catalytic nucleic acid is a DNAzyme.

10

44. The isolated catalytic nucleic acid of claim 43, wherein said catalytic core sequence comprises the sequence set forth in SEQ ID NO:68.

15 45. An isolated, single-stranded nucleic acid molecule consisting of between five and 40 nucleotides, wherein said nucleic acid molecule comprises a sequence complementary to at least five consecutive nucleotides of a sequence selected from the group consisting of SEQ ID NOs:4-48.

20 46. The nucleic acid molecule of claim 45, wherein said nucleic acid molecule is DNA.

47. An isolated, double-stranded RNA molecule, wherein one strand of said RNA molecule comprises a nucleic acid sequence complementary to at least five consecutive nucleotides of a sequence selected from the group consisting of SEQ ID NOs:4-48.

25

48. The RNA molecule of claim 47, wherein said RNA molecule consists of between five and 40 nucleotides.

30 49. The RNA molecule of claim 47, wherein said RNA molecule consists of between 18 and 25 nucleotides.

50. A method for making a library of single-stranded DNAzymes, said method comprising:

- (a) amplifying nucleic acid in an amplification reaction to form an amplification reaction product, wherein said amplification reaction comprises template nucleic acid and a primer containing at least one ribose nucleotide, wherein one strand of said template nucleic acid comprises said library of single-stranded DNAzymes, and wherein said amplification reaction product comprises double-stranded nucleic acid with a strand comprising said at least one ribose nucleotide,
- (b) contacting said amplification reaction product with a base hydrolysis agent under conditions wherein said strand comprising said at least one ribose nucleotide becomes shorter than the other strand of said double-stranded nucleic acid, and
- (c) obtaining the strand from step (b) comprising said library of single-stranded DNAzymes based on size.

51. The method of claim 50, wherein said amplification reaction further comprises a primer lacking ribose nucleotides.

52. The method of claim 50, wherein said amplification reaction comprises two primers comprising at least one ribose nucleotide.

53. The method of claim 50, wherein the shorter strand of said double-stranded nucleic acid of step (b) comprises said library of single-stranded DNAzymes.

54. The method of claim 50, wherein the longer strand of said double-stranded nucleic acid of step (b) comprises said library of single-stranded DNAzymes.

55. The method of claim 50, wherein said base hydrolysis agent is sodium hydroxide.

56. A composition comprising a nucleic acid molecule and a keratolytic agent, wherein said nucleic acid molecule comprises a sequence complementary to a nucleic acid sequence present in an HPV, and wherein said keratolytic agent is present in an

amount effective to enhance penetration of said nucleic acid molecule.

57. The composition of claim 56, wherein said nucleic acid molecule is an antisense oligonucleotide or an siRNA molecule.

5

58. The composition of claim 56, wherein said nucleic acid molecule is a ribozyme or DNAzyme.

59. The composition of claim 56, wherein said keratolytic agent is salicylic acid.

10

60. A nucleic acid molecule comprising  $(N)_{n1}$  followed by (oxygen-carbon-carbon) $_{n2}$  followed by  $(N)_{n3}$ , wherein N is a nucleotide, and wherein said  $n1$ ,  $n2$ , and  $n3$  are integers greater than 0.

15

61. The nucleic acid molecule of claim 60, wherein said  $n1$  is between 10 and 20.

62. The nucleic acid molecule of claim 60, wherein said  $n2$  is between 3 and 10.

63. The nucleic acid molecule of claim 60, wherein said  $n3$  is between 10 and 20.

20

64. The nucleic acid molecule of claim 60, wherein said  $(N)_{n1}$  is a DNA sequence of 17 consecutive adenosines.

65. The nucleic acid molecule of claim 60, wherein said  $(N)_{n3}$  is a 5'-

25

TGTAACGACGGCCAG-3' sequence.

66. The nucleic acid molecule of claim 60, wherein said nucleic acid molecule comprises a phosphate group between said (oxygen-carbon-carbon) $_{n2}$  and said  $(N)_{n3}$ .

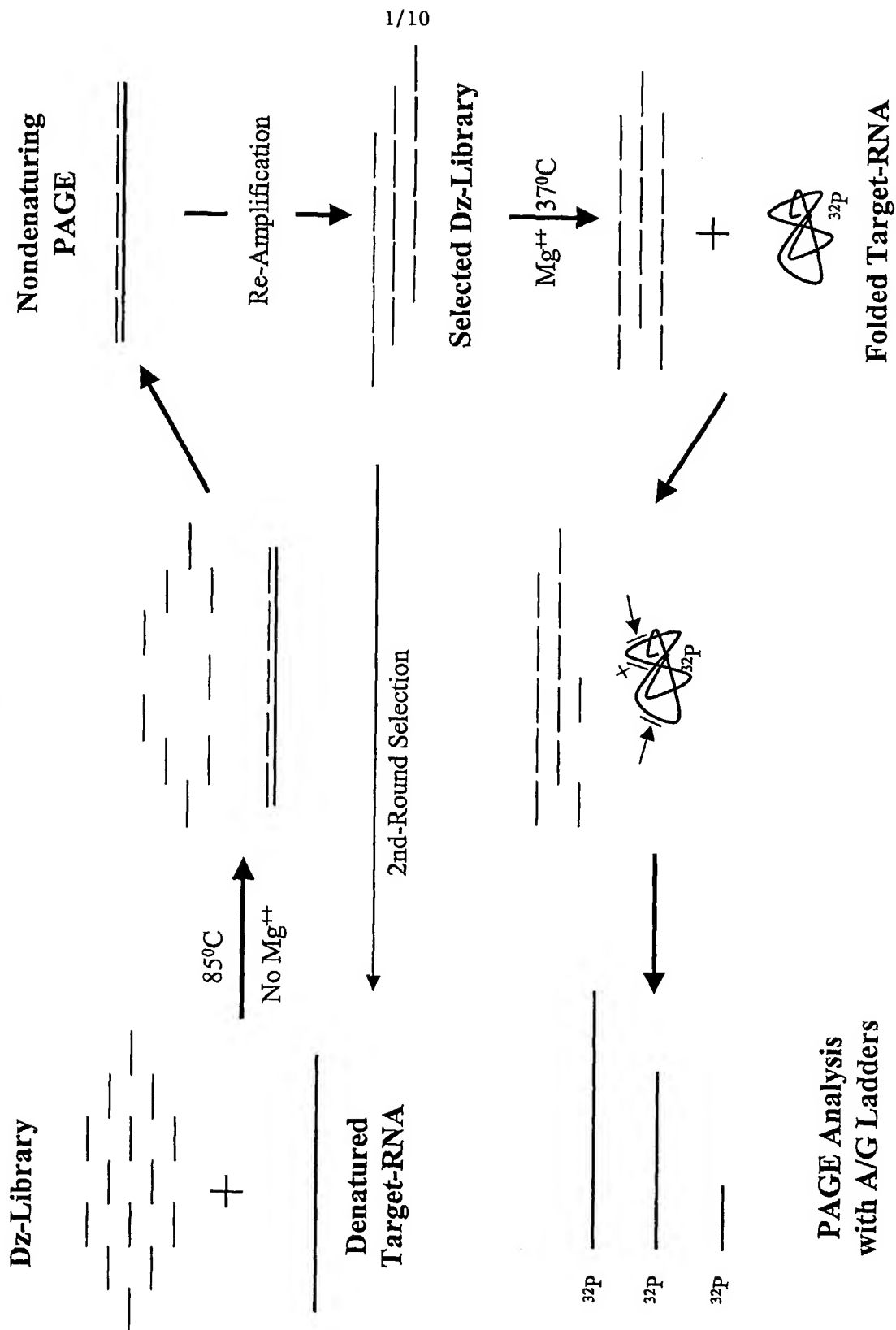
30

67. A method for making a library of single-stranded DNAzymes, said method comprising:

- (a) amplifying nucleic acid in an amplification reaction to form an amplification reaction product, wherein said amplification reaction comprises template nucleic acid and a primer comprising an (oxygen-carbon-carbon)<sub>n</sub> backbone unit, wherein n is an integer greater than 1, wherein one strand of said template nucleic acid comprises  
5 said library of single-stranded DNAzymes, and wherein said amplification reaction product comprises double-stranded nucleic acid wherein the strand extending from said primer is shorter than the other strand of said double-stranded nucleic acid, and
- (b) obtaining the strand from step (a) comprising said library of single-stranded DNAzymes based on size.

10

**Figure 1**





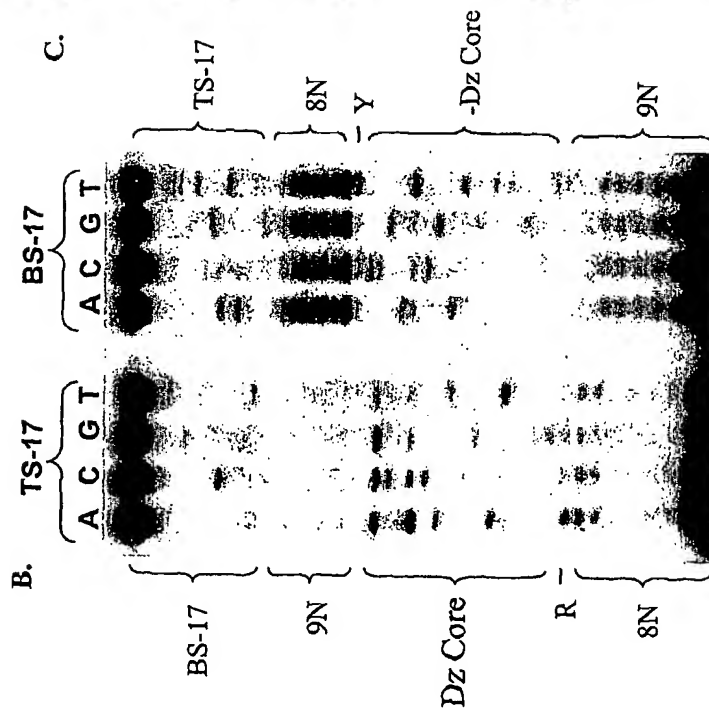
# Figure 2

A.

TS-17 Primer  
 5' - CAGGAAACAGCTATGAC - 3'  
 5' - CAGGAAACAGCTATGACNNNNNNNNRGGCTAGCTACAAACGANNNNNNNNCTGGCCGTCGTTTACA - 3'  
 3' - GACCGGCAGCAAAATGT - 5'

BS-17 Primer  
 5' - CAGGAAACAGCTATGACNNNNNNNNRGGCTAGCTACAAACGANNNNNNNNCTGGCCGTCGTTTACA - 3'  
 3' - GTCCTTGTGCGATACTGNNNNNNNNYCCGATCGATGTTGCTNNNNNNNGACCGGCAGCAAAATGT - 3'

PCR ↓



C.

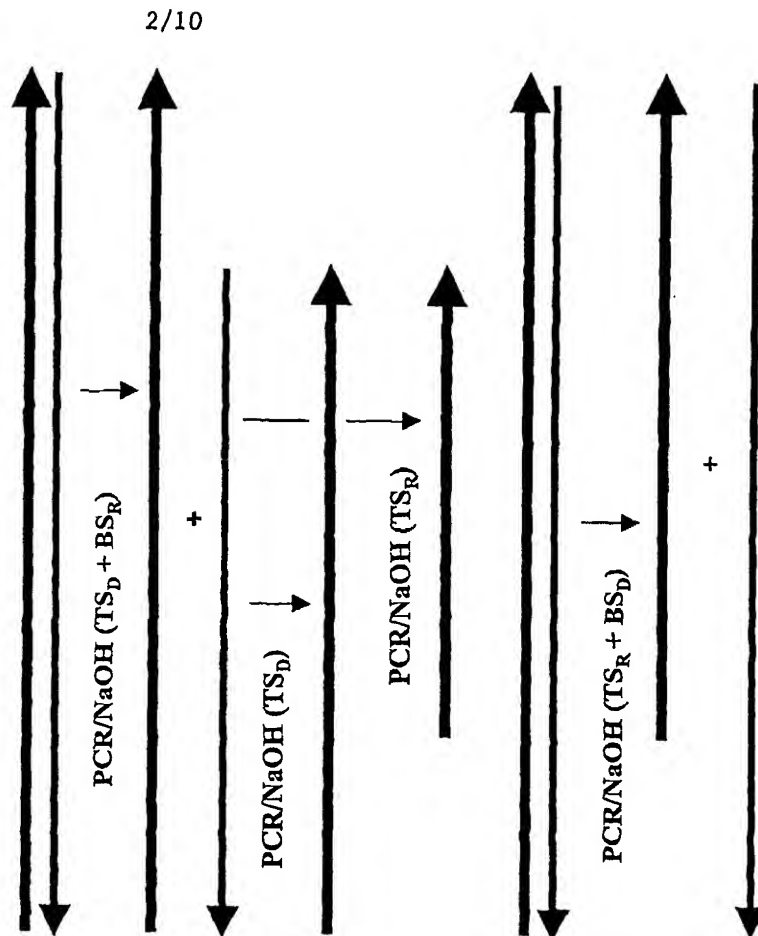


Figure 3

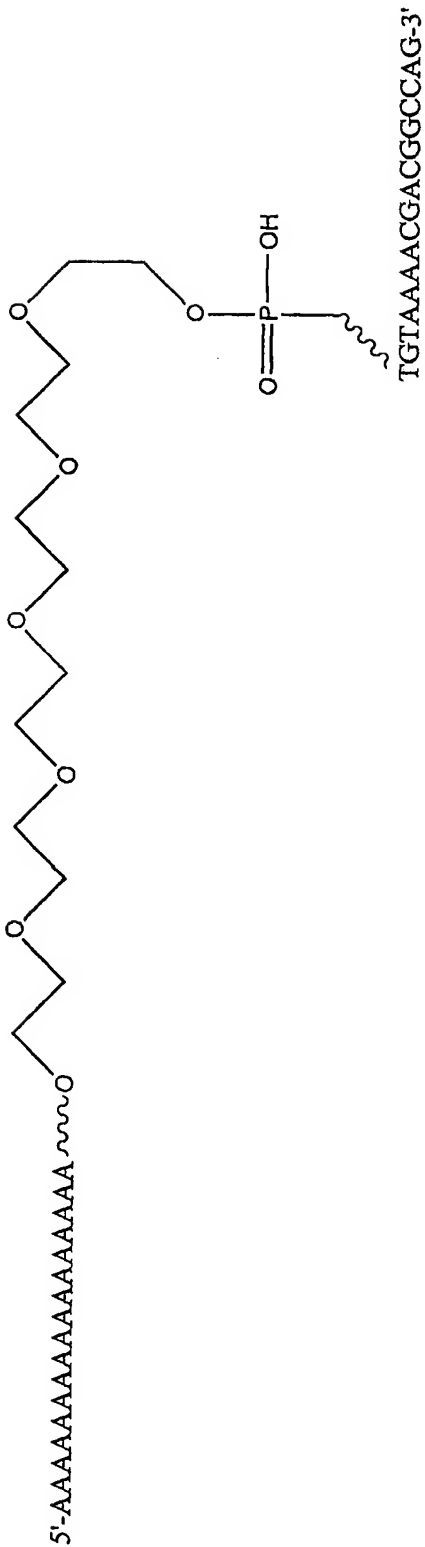
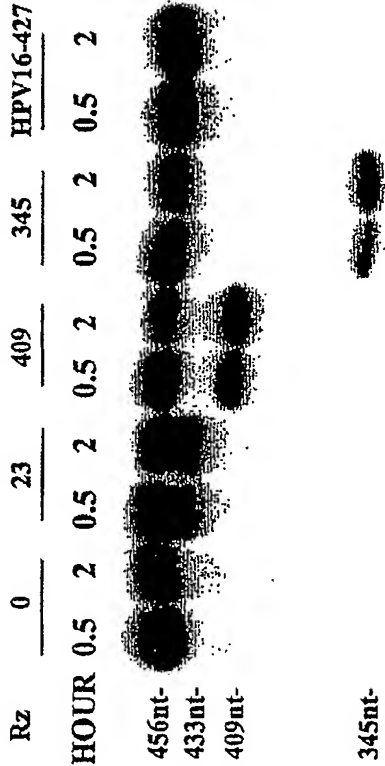
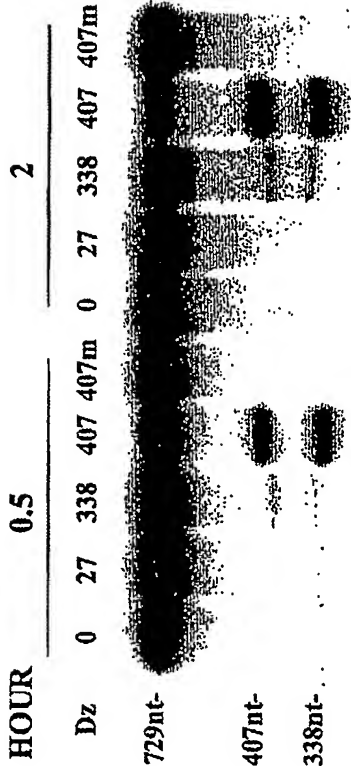


Figure 4

A.



B.



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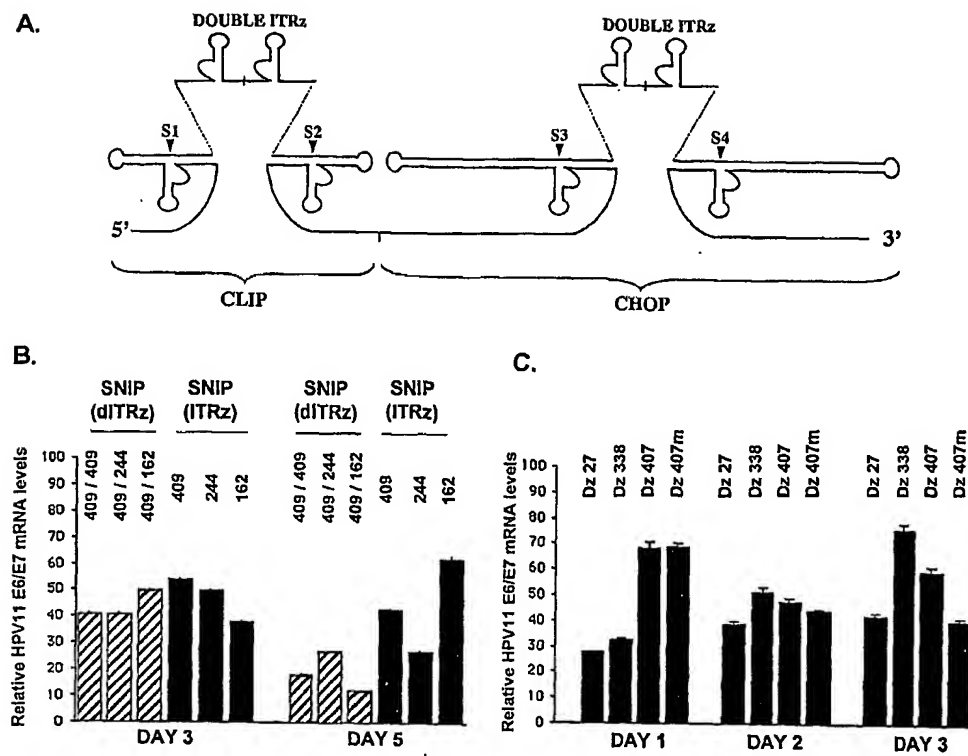
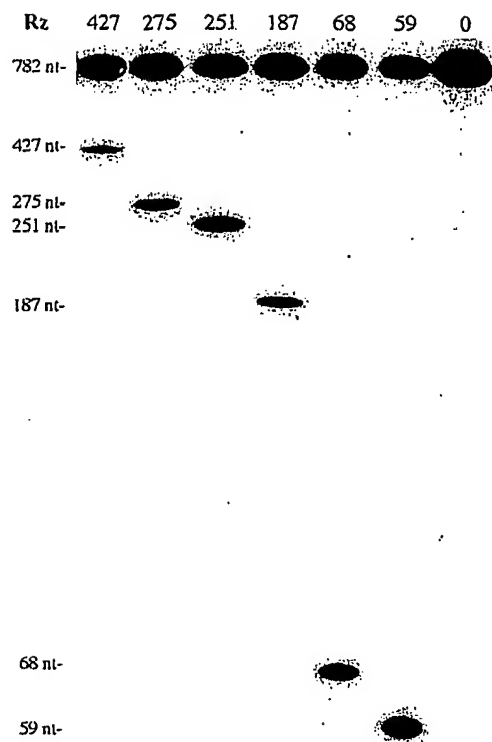


Figure 5

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A.



B.

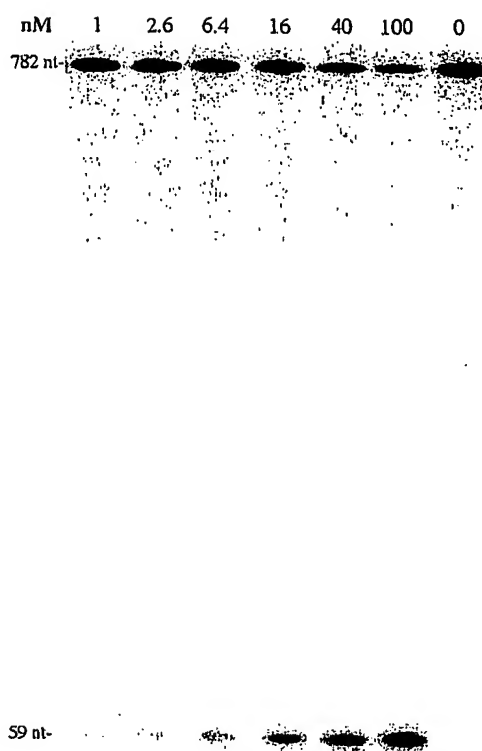


Figure 6

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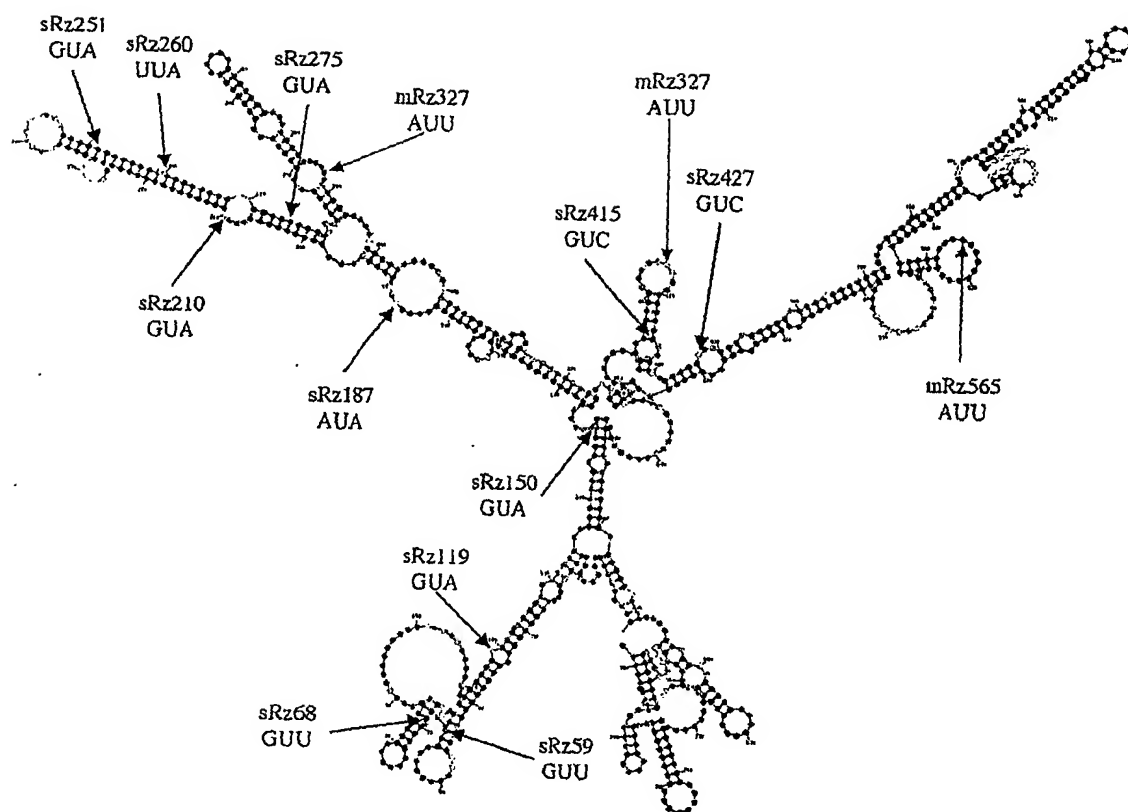
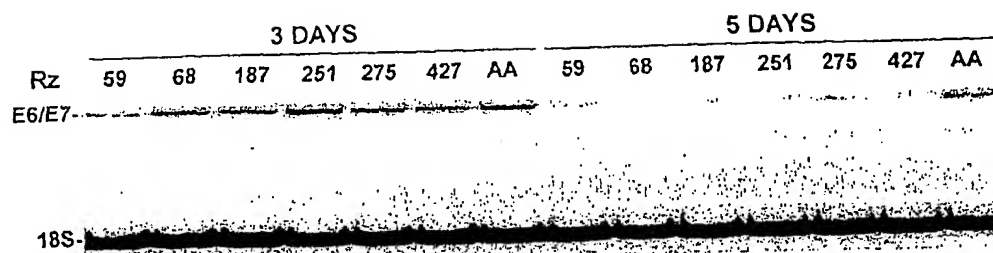


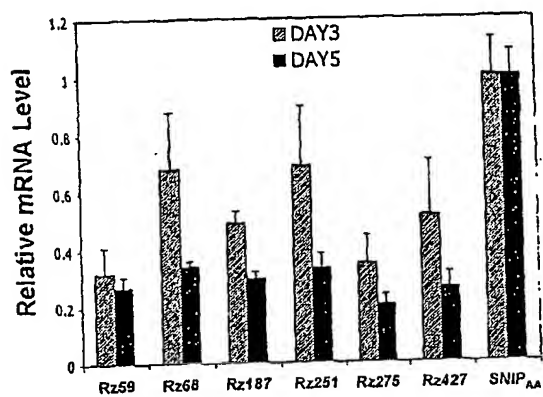
Figure 7

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A.



B.



C.

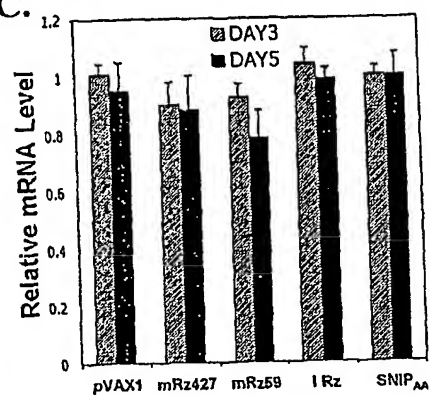
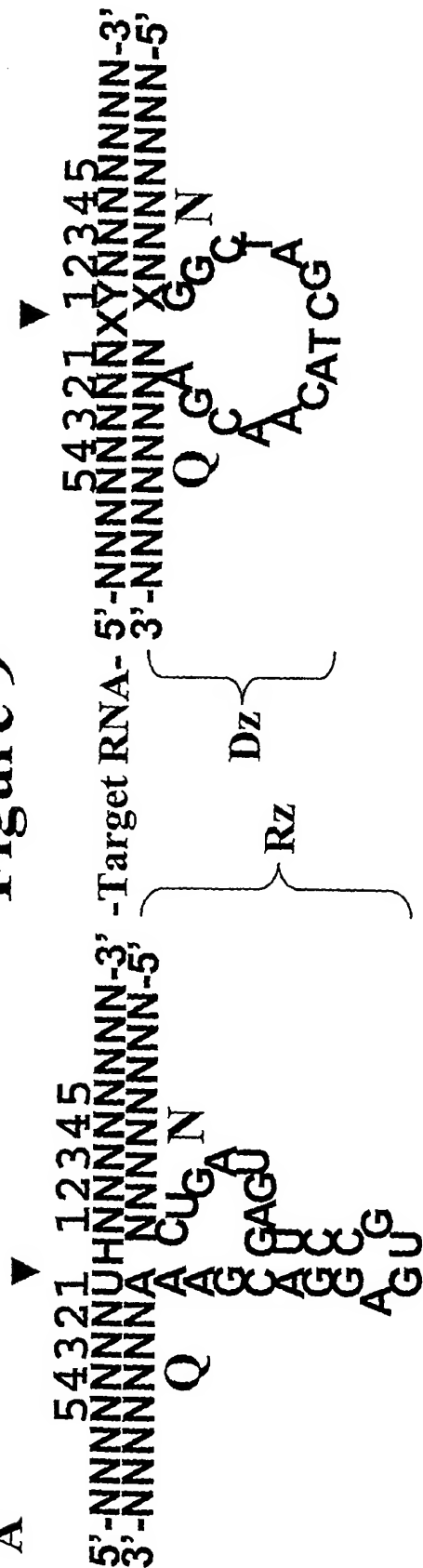


Figure 8

Figure 9

A



H=A, C, U

X=A, G, C, T; Y=A, G, C, T

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B

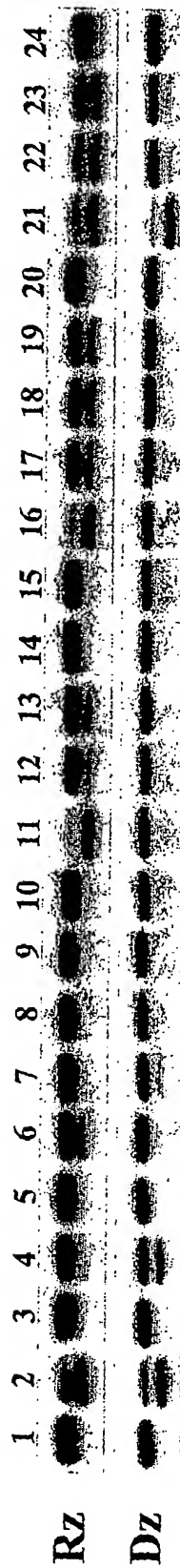
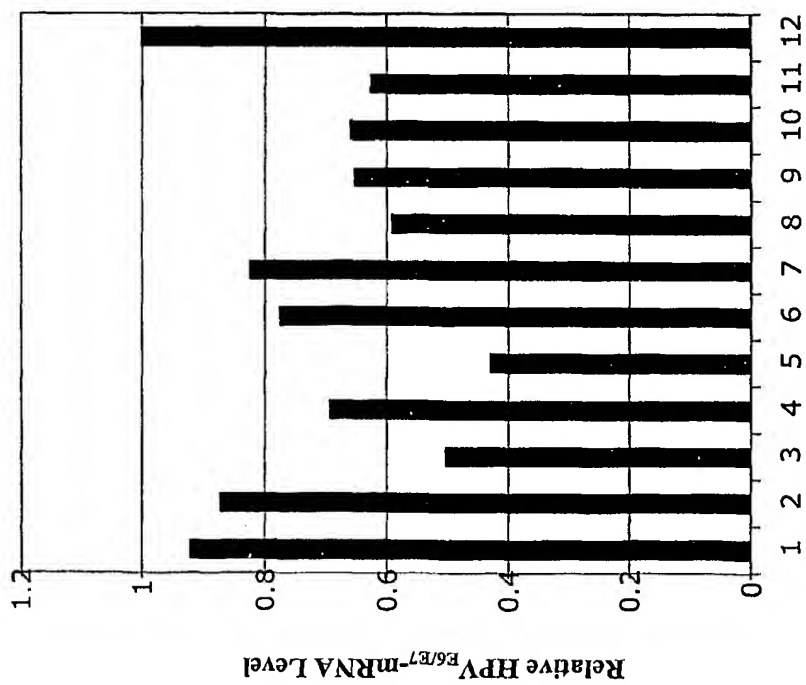


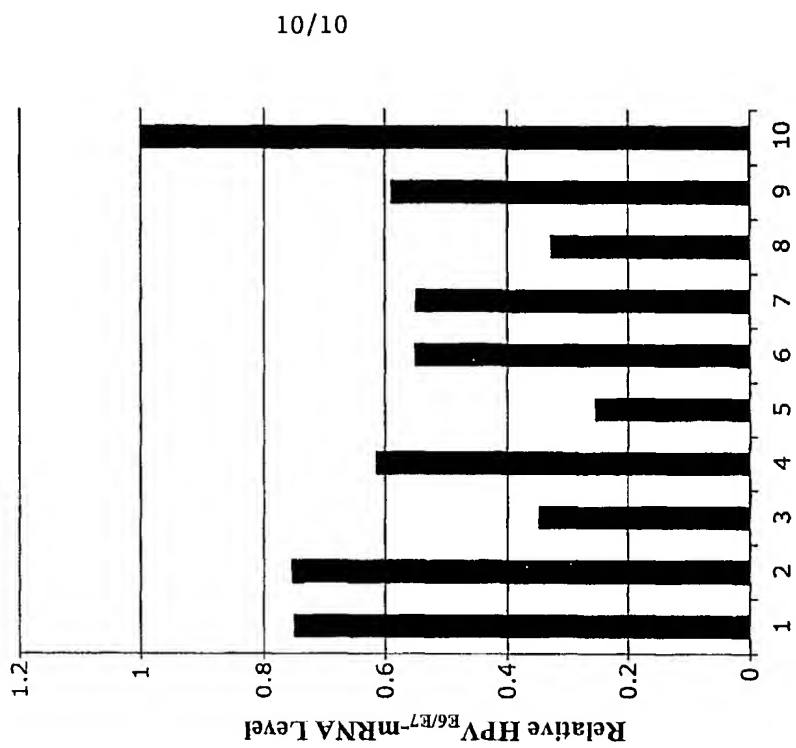


Figure 10

A



B



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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/20340

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 48/00; C12N 15/00; C07H 21/04, 21/02

US CL : 435/375, 377; 536/23.1, 24.1, 24.5

According to International Patent Classification (IPC) or to both national classification and IPC

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Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/375, 377; 536/23.1, 24.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
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## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 00/09673 A1 (JOHNSON & JOHNSON RESEARCH PTY LIMITED) 24 February 2000(24.02.2000), see entire document. but especially Figure 1 for SEQ ID NO: 4 and claim for SEQ ID NO: 68.	1-19, 40-44, 50-55
Y	WO 00/60115 A2 (CITY OF HOPE) 12 October 2000(20.10.2000), see entire document, but especially page 6 for SEQ ID NO: 51.	1-19, 40-44, and 50-55
Y	US 6,326,174 B1 (JOYCE et al.) 04 December 2001, see entire document.	1-19, 40-44, and 50-55
Y	US 6,174,870 B1 (CROOKE et al.) 16 January 2001(16.01.2001), see entire document.	1-19, 40-44, and 50-55
Y	LIEBER et al. Selection of Efficient Cleavage sites in Target RNAs by Using a Ribozyme Expression Library. Molecular and Cellular Biology. January 1995, Vol. 15, No. 1, pages 540-551, see entire document.	1-19, 40-44 and 50-55
Y	CHEN et al. Effectiveness of Three Ribozymes for Cleavage of an RNA Transcript from Human Papillomavirus Type 18. 1995, Vol. 2, No. 4, pages 263-271, see entire document.	1-19, 40-44, and 50-55

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/20340

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This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.: 1-19, 40-44 (SEQ ID NOS: 4, 51, and 68), and 50-55
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

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